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Complement Protein C1q Directs Macrophage Polarization and Limits Inflammasome Activity during the Uptake of Apoptotic Cells

Marie E. Benoit, Elizabeth V. Clarke, Pedro Morgado, Deborah A. Fraser, and Andrea J. Tenner

Deficiency in C1q, the recognition component of the classical complement cascade and a pattern recognition receptor involved in apoptotic cell clearance, leads to lupus-like autoimmune diseases characterized by auto-antibodies to self proteins and aberrant innate immune cell activation likely due to impaired clearance of apoptotic cells. In this study, we developed an autologous system using primary human lymphocytes and human monocyte-derived macrophages (HMDMs) to characterize the effect of C1q on macrophage gene expression profiles during the uptake of apoptotic cells. C1q bound to autologous apoptotic lymphocytes modulated expression of genes associated with JAK/STAT signaling, chemotaxis, immunoregulation, and NLRP3 inflammasome activation in LPS-stimulated HMDMs. Specifically, C1q sequentially induced type I IFNs, IL-27, and IL-10 in LPS-stimulated HMDMs and IL-27 in HMDMs when incubated with apoptotic lymphocyte conditioned media. Coincubation with C1q tails prevented the induction of type I IFNs and IL-27 in a dose-dependent manner, and neutralization of type I IFNs partially prevented IL-27 induction by C1q. Finally, C1q decreased procaspase-1 cleavage and caspase-1-dependent cleavage of IL-1β suggesting a potent inhibitory effect of C1q on inflammasome activation. These results identify specific molecular pathways induced by C1q to suppress macrophage inflammation and provide potential therapeutic targets to control macrophage polarization and thus inflammation and autoimmunity. The Journal of Immunology, 2012, 188: 000–000.

The complement system, a powerful effector of the innate immune system, consists of a group of proteins circulating as inactive precursors in the blood and in extracellular fluids. Upon activation through the classical, lectin, or alternative pathway, a cascade of proteolytic cleavages and formation of central enzymatic complexes (C3 and C5 convertases) leads to the generation of active fragments resulting in the opsonization of invading pathogens (C1q, C3b, and iC3b), release of proinflammatory chemotactic factors (C3a and C5a), which recruit leukocytes to the site of infection or injury, and finally formation of the membrane attack complex (C5b-9) and subsequent lysis of the pathogen (1, 2). Complement functions as an important humoral defense system to sense danger by recognizing pathogen-associated molecular patterns (PAMPs) but is also activated by damage-associated molecular patterns (DAMPs) or altered self tissues. Dysregulated complement activation has been associated with the development of various diseases including rheumatoid arthritis and Alzheimer’s disease (2, 3). A causal link between complement deficiency and systemic lupus erythematosus (SLE) involves in part the role of complement in physiological waste disposal mechanisms, in particular clearance of dying cells (4). Although activation by all three complement pathways can contribute to enhanced uptake of apoptotic cells by phagocytic cells (5–8), homozygous deficiency of any of the early complement components of the classical pathway (C1q, C1r, C1s, C4, and C2) predisposes to the development of SLE with >90% of individuals with genetic deficiency of C1q developing severe SLE (9).

C1q is known to play a prominent nonredundant tissue-specific role in the clearance of apoptotic cells in vitro and in vivo (10–14). C1q binds to apoptotic cells and cellular debris through its globular heads (10, 15) and to phagocytic receptors through its collagen tails (1, 16). Although at first thought to be primarily of liver origin, C1q is predominantly synthesized in vivo by peripheral tissue macrophages and dendritic cells (17, 18) and by myeloid cells in vitro (8, 19–21). Although C1q is most often bound to C1r and C1s in the circulation (22), this local synthesis of C1q is hypothesized to be the major source of C1q for the rapid opsonization of dying cells in tissue before recruitment of plasma-derived components such as C1r and C1s and subsequent activation of the complement cascade. In addition, induced synthesis of C1q has been detected in several injury models in vivo and in vitro(23, 24); reviewed in Ref. 3), suggesting that the induction of C1q synthesis in tissue may be a response to injury that promotes rapid clearance of apoptotic cells and concomitant suppression of inflammation. For example, interaction of C1q with human monocytes or dendritic cells results in the downregulation of proinflammatory cytokines.
C1q LIMITS MACROPHAGE AND INFLAMMASOME ACTIVATION

Upon TLR4 stimulation by LPS (25, 26). Recently, we showed that C1q enhances uptake of apoptotic Jurkat T cells by human monocytes but has no effect on the basal clearance level of these apoptotic cells by human monocyte-derived macrophages (HMDMs) and dendritic cells (8). In addition, although C1q influences the induction of cytokines in all myeloid cell types tested in this study, both the degree and direction of modulation depend on the state of differentiation of the phagocytic cell (8). However, because several C1q receptors have been identified and none has been shown specifically to mediate C1q-enhancement of phagocytosis of apoptotic cells (1, 12, 27), the intracellular signaling pathways engaged upon interaction of C1q with phagocytic cells remain to be fully elucidated. In addition, because characterization of macrophage activation in response to C1q has been limited to the study of few candidate cytokines, chemokines, and/or signaling molecules, the extent of the effect of C1q on macrophage polarization and inflammatory responses during uptake of apoptotic cells remains largely uncharacterized. In this study, we developed a unique system using primary human autologous lymphocytes and HMDMs to characterize the effect of C1q on macrophage gene expression profiles during the uptake of autologous apoptotic cells, a more physiologic system than transformed cell lines as a source of apoptotic cells. Our results show that C1q bound to autologous apoptotic lymphocytes (ALs) significantly modulates the response of HMDMs to LPS by increasing expression of cytokines, chemokines, and effector molecules associated with immunoregulation and by directly suppressing caspase-1–dependent cleavage of IL-1β, in absence of any other complement proteins.

Materials and Methods

Media and reagents

RPMI 1640, penicillin/streptomycin, trypsin–EDTA, and L-glutamine were from HyClone. Recombinant human M-CSF and recombinant human IL-2 were from PeproTech. ATnP was from Sigma-Aldrich. Mouse IgG Abs were from R&D Systems, and anti-human IFN-α and -β Abs were from PBL Biomedical Laboratories. Human serum albumin used for elutriation was obtained from Talcercis Biotherapeutics. Ultrapure LPS was from List Biological Laboratories. C1q was isolated from plasma-derived normal human serum by ion-exchange chromatography, followed by size-exclusion chromatography according to Tenner et al. (28) and modified as described (29). C1q tails were prepared as described (30). All C1q preparations showed equivalent purity (determined by SDS-PAGE and Coomassie staining) and have <0.03 endotoxin units/ml endotoxin by Limulus amebocyte lysate clot assay (Lonza).

Cell isolation and culture

All blood samples were collected into CPDA1 at the University of California, Irvine, Institute for Clinical and Translational Science in accordance with guidelines and approval of the University of California, Irvine, Institutional Review Board. Human PBLS and monocytes were isolated by counterflow elutriation using a modification of the technique of Lionetti et al. (31) as described previously (32). Cell purity was determined by standard flow cytometry on a FACSCalibur (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star). About 80% of the cells of the lymphocyte fraction were CD3+ (Supplemental Fig. 1A), and >90% of the monocyte fraction was CD11b+. Lymphocytes were maintained for 7 d in RPMI 1640, 10% FCS, 2 mM t-glutamine, and 1% penicillin/streptomycin (complete media) containing 50 U/ml recombinant human IL-2 and then gamma-irradiated (10 Gy) and maintained overnight in complete media or media without FBS to generate early apoptotic lymphocytes [EALs; annexin V+/propidium iodide (PI)−] or late apoptotic lymphocytes (LALs; annexin V+/PI+), respectively (Supplemental Fig. 1B). In some experiments, lymphocytes were preabeled with a red membrane cell tracker (PKH26; Sigma-Aldrich) according to the manufacturer’s instructions. HMDMs were generated from monocytes by culture for 8 d in complete media containing 25 ng/ml recombinant human M-CSF. For every experiment, apoptosis (apoptosis detection kit from BioVision) and HMDM phenotype (Supplemental Fig. 2A) were assessed by flow cytometry.

C1q binding assay

EALs and LALs were incubated with 150 μg/ml purified human C1q for 1 h in PBS/1% human serum albumin at 37°C. Binding of C1q was assessed for every experiment by flow cytometry using an mAb against C1q (Quidel) and FITC–anti-mouse IgG (Jackson ImmunoResearch Laboratories). For each experiment, C1q binding was >50% for EALs or LALs.

Uptake assay

PKH26-labeled or unlabeled EALs and LALs, precoated or not with C1q, were incubated with HMDMs at a 5:1 ratio for 1 h (optimal ratio and time determined in preliminary experiments, see Supplemental Fig. 2B) in phagocytosis buffer (RPMI 1640, 25 mM HEPES, and 5 mM MgCl2). For uptake quantification, cells were washed, harvested with trypsin/EDTA, and stained with CD11c–FITC Abs for flow cytometry analysis or fixed with 3.7% paraformaldehyde and stained with FITC–phalloidin (Invitrogen) according to the manufacturer’s instructions. For confocal imaging, cells were analyzed using the Nikon Ti microscope and the EZ C1 software. Images were analyzed using Adobe Photoshop CS and ImageJ.

RNA extraction and microarray analysis

After uptake, HMDMs were stimulated with 10 ng/ml LPS (each condition performed in triplicate) for 3 h in serum-free HL-1 media. Total RNA was extracted using the IllustraRnAspin Mini Isolation Kit (GE Healthcare). Gene expression profiles were studied using the Human Gene 1.0 ST array (Affymetrix). RNA labeling and hybridization were performed according to the manufacturer’s instructions by the University of California, Irvine, Genomics High Throughput Facility. Data processing and analysis were performed using JMP Genomics 5.0 software (SAS Institute). Briefly, interarray median correction was used to normalize signal intensities. Significant differences in gene expression compared with unstimulated HMDMs were identified by ANOVA test using Holm multiple testing method and a false-positive rate (α error) of 0.05 (see Supplemental Table 1 for a complete list of modulated genes). Hierarchical clustering (Pearson correlation coefficient-based heat map using complete linkage method) was performed using JMP genomics and TMeV (33). Functional classification was performed using DAVID software (http://david.abcc.ncifcrf.gov/) (34), and pathway network analysis and visualization was performed using Cytoscape (35). All data were submitted to the MIAME-compliant database Gene Expression Omnibus (accession number GSE30177; http://www.ncbi.nlm.nih.gov/geo/).

Reverse transcription and quantitative real-time PCR

The cDNA synthesis was carried out with 100 ng of total RNA and the M-MLV reverse transcriptase (Invitrogen) as previously described (37). Quantitative PCR was performed using the Maxima SYBR/Green Master Mix (Thermo Fisher Scientific), the iCycler iQ, and the iQ5 software (Bio-Rad). The fold-change (FC) was determined as follows: $FC = 2^\Delta\triangle Ct$, where $\Delta\triangle Ct = (Ct_{Target} - Ct_{GAPDH})_{Unstimulated} - (Ct_{Target} - Ct_{GAPDH})_{Unstimulated}$.

Cytokine secretion assays

Human IFN-α detection kit was from Mabtech, and IL-27 and IL-10 human detection kits were from BioLegend. ELISAs were performed according to the manufacturer’s instructions.

Detection of cleaved caspase-1

After uptake, HMDMs were washed and stimulated with 10 ng/ml LPS in HL-1 media for 6 h. ATP (1 mM) was added to the cell culture 90 min before the end of LPS stimulation. HMDMs were then incubated with Green FLICA Caspase-1 probes (ImmuNoChemistry Technologies) and Cell Tracker Blue CM-ujC (Invitrogen) 1 h before the end of the stimulation. HMDMs were then washed, fixed, and analyzed immediately by confocal microscopy as described above.

Western blot

For detection of inflammasome components, HMDMs were stimulated with 10 ng/ml LPS for 6 h, and 1 mM ATP was added for the last 1 h of stimulation. For detection of IL-1β, HMDMs were stimulated with LPS for 18 h, and ATP was added during the last 3 h of LPS stimulation. HMDM culture supernatants were concentrated using 10 KDa Amicon columns (Millipore). HMDMs were harvested in RIPA lysis buffer. Proteins were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes (GE Healthcare). Immunoblots were performed using primary
Abs against NLRP3 (Enzo Life Sciences), caspase-1 (Cell Signaling), apoptosis-associated speck-like protein containing a CARD domain (ASC; Medical & Biological Laboratories), IL-1β (clone 32D from the National Cancer Institute Biological Resources Branch), and β-actin (Sigma-Aldrich) and secondary HRP-conjugated anti-mouse or rabbit IgG Abs (Jackson ImmunoResearch Laboratories). The blots were developed using ECL plus (GE Healthcare) and analyzed using the Nikon D700 digital SLR camera and the ImageJ software as described (39).

**Statistical analysis**

Results were calculated as means ± SD and compared with two-way ANOVA followed by Bonferroni post hoc test, α error = 0.05, using GraphPad Prism (unless otherwise stated, all conditions are compared with unstimulated HMDMs). Differences were considered significant when the p value was <0.05.

**Results**

**C1q binds to human ALs and enhances their ingestion by HMDMs**

To determine how C1q modulates human macrophage responses during the uptake of apoptotic cells, we developed a unique autologous system (lymphocytes and monocytes being isolated from the same donor) where HMDMs were incubated with C1q-coated ALs. Lymphocytes were irradiated to induce apoptosis and maintained overnight in complete media or under serum deprivation to generate early (EAL; annexin V+/PI−) and late (LAL; annexin V+/PI+, secondary necrosis stage) ALs, respectively (Supplemental Fig. 1B). In preliminary experiments (Supplemental Fig. 1D, 1E), the optimal C1q concentration (150 μg/ml) and incubation time (1 h) to obtain the highest percentage of C1q binding to ALs while maintaining C1q concentration at near physiological levels was determined (C1q serum concentration is ∼113 ± 40 μg/ml, ranging from 56 to 276 μg/ml depending on the studies and methods (40, 41)]. We found that C1q binds to human EALs and LALs with the same efficiency, as ∼55–60% of both EALs and LALs were C1q positive and have comparable mean fluorescence intensity (Fig. 1A, 1B). Prelabelling of EALs and LALs with the red cell membrane tracker PKH26 did not affect the induction of apoptosis (Supplemental Fig. 1C) or C1q binding to those cells (Supplemental Fig. 1F). The effect of C1q on the uptake of EALs and LALs by HMDMs was then determined by immunocytochemistry and flow cytometry (Supplemental Fig. 2B, 2C and Fig. 1C, 1D). It is of note that all uptake assays and subsequent stimulation were performed in serum-free medium so no complement proteins other than C1q are present in this system to mimic the tissue environment (early in injury or during homeostatic apoptotic cell clearance) before the recruitment of plasma-derived complement proteins. C1q significantly (p < 0.05) increased the percentage of HMDMs that have ingested at least one EAL but did not increase the percentage of HMDMs that have ingested at least one LAL (Fig. 1C, 1D). However, C1q increased the number of EALs and LALs per HMDM, particularly the percentage of HMDMs that have ingested three or more EALs (p < 0.01, Fig. 1C) or LALs (p < 0.05, Fig. 1C). Altogether, these results showed that C1q binds with the same efficiency to EALs and LALs and enhances their uptake by HMDMs.

To determine if HMDMs preferentially ingest those EALs and LALs bound to C1q, we quantified the number of C1q-positive cells after uptake by immunostaining (Fig. 1E, 1F). C1q was detectable on EALs or LALs during the uptake by HMDMs (Fig. 1E), and more than 70% of macrophage-bound EALs (72.1 ± 17.6%, p < 0.05) or LALs (74.1 ± 12.3%, p < 0.01) were C1q positive (Fig. 1F). Altogether, these results suggest that C1q remained on EAL and LAL surfaces acting as a powerful “eat-me” signal to enhance the uptake and therefore may directly signal macrophages to modulate their responses during phagocytosis.

**C1q modulates expression of genes associated with chemotaxis, inflammation, signaling, and NLRP3 inflammasome activation in LPS-stimulated HMDMs during the uptake of ALs**

To delineate the C1q-modulated pathways in macrophages during the uptake of ALs, HMDMs were incubated with C1q-bound EALs or LALs for 1 h and then stimulated for 3 h with a low dose of LPS (10 ng/ml). LPS is used as a tool in this system to mimic local inflammation induced by DAMPs such as through activation of TLRs (such as TLR4) by high mobility group box 1 (HMGB1) or heat shock proteins (HSPs) that are normally intracellular but are released when the cells die (42). Global transcriptional gene expression profiles of HMDMs were analyzed using the Gene 1.0ST array from Affymetrix (Fig. 2 and Supplemental Fig. 3) and validated by quantitative real-time PCR (qRT-PCR) (Supplemental Fig. 3). Hierarchical clustering analysis highlighted groups of genes specifically modulated by the uptake of EALs and LALs compared with LPS alone and also by C1q when bound to EALs or LALs compared with EALs or LALs in the absence of C1q (Fig. 2A). Using gene ontology (GO) annotation, genes modulated by C1q showed enriched GO biological processes related to signal transduction, G protein-coupled receptor signaling pathway, immune response, homeostasis, and macromolecular biosynthesis (Fig. 2B). Network pathway analysis of these biological processes using Cytoscape showed that C1q modulated genes associated with chemotaxis, inflammation/cytokines, JAK/STAT signaling, and NLRP3 inflammasome activation (Fig. 2C–F and Supplemental Table I), some of those genes being differentially modulated when C1q is bound to EALs or LALs suggesting that the apoptotic cell stage influences the C1q effect on macrophage response. For example, C1q increased the expression of the chemokine CX3CL1 (fractalkine) and the chemokine receptor CCR3 in HMDMs only when bound to EAL (Fig. 2C). When bound to LAL, C1q significantly increased the expression of versican, IL-13, a typical M2-driven cytokine, and IL-1F7, also known as IL-37, while decreasing the expression of the M1-associated chemokine CXCL9 in HMDMs (Fig. 2C, 2D). C1q bound to either EAL or LAL decreased the expression of VEGF-C and increased the expression of immunoregulatory and immunosuppressive cytokines such as IL-33, IL-27, and the type I IFNs receptor IFNAR2 in HMDMs (Fig. 2C, 2D). It is important to note that no IL-27 mRNA, the most upregulated gene by C1q, was detectable in C1q-bound EALs or LALs thus eliminating any possibilities of RNA contamination from ALs in our microarray results (Supplemental Fig. 3G). In addition, C1q bound to EALs and LALs modulated the expression of several signaling molecules of the JAK/STAT pathway (Fig. 2E). Finally, C1q bound to EALs and LALs increased the expression of NLRP12, a negative regulator of NF-κB and inflammasome activation (Fig. 2F). When bound to LALs, C1q decreased the expression of NLRP3/NALP3 (also downregulated by EALs alone by 2-fold with no further effect of C1q on this downregulation; see Supplemental Table I), one of the main components of the NLRP3 inflammasome, a cytosolic protein complex formed by the association of NLRP3, procaspase-1, and ASC that cleaves procaspase-1 to generate active caspase-1. At the same time, C1q bound to LALs increased the expression of POP1/ASC2 (Fig. 2F), a pyrin domain containing protein that associates with ASC and may destabilize the NLRP3 inflammasome. Altogether, these results suggest that C1q promotes the expression of potent immunoregulatory and immunosuppressive cytokines and negatively regulates NLRP3 inflammasome activation.

C1q increases type I IFNs, IL-27, and IL-10 secretion by LPS-stimulated HMDMs during the uptake of ALs

LPS stimulation of mouse macrophages induces IL-10 production through the sequential induction of type I IFNs and IL-27 (43).
Because IL-27 was one of the most upregulated cytokines by C1q bound to EALs and LALs in HMDMs (~4-fold increase compared with unstimulated HMDMs and 2-fold increase compared with EALs or LALs alone, Fig. 2D and Supplemental Table I) and IFNA6 mRNA was slightly upregulated by C1q in HMDMs (Supplemental Table I), we investigated if C1q modulates type I
IFNs, IL-27, and IL-10 expression in human macrophages. C1q transiently increased IFN-α and IFN-β mRNA levels (Fig. 3A–D) in HMDMs, and the secretion of IFN-α by HMDMs was significantly ($p < 0.001$) increased by C1q bound to EALs or LALs after 6 h of LPS stimulation (Fig. 3A, 3B, insets). C1q bound to EALs and LALs significantly increased IL-27 mRNA levels after 3, 6, and 12 h of LPS stimulation (Fig. 3E, 3F), and the IL-27 protein levels were significantly ($p < 0.001$) increased by C1q–EALs and C1q–LALs after 18 h of LPS stimulation (Fig. 3E, 3F, insets). Notably, the levels of IL-27 were decreased by LPS in a dose-dependent manner, and the effect of C1q on IL-27 expression was totally inhibited after stimulation with higher dose of LPS (Fig. 3I). Finally, C1q bound to EALs and LALs significantly ($p < 0.05$) increased IL-10 mRNA levels in HMDMs after 18 h of LPS stimulation (Fig. 3G, 3H). The protein levels of IL-10 were also increased by C1q after 18 h of LPS stimulation compared with LPS ($p < 0.05$) and EALs or LALs (Fig. 3G, 3H, insets).

C1q bound to apoptotic cells synergizes with apoptotic cell signals in inducing IL-27 in HMDMs during the uptake of ALs

We next investigated whether C1q modulates type I IFNs, IL-27, and/or IL-10 in resting HMDMs (i.e., non-LPS-stimulated). HMDMs were incubated with C1q-bound EALs or LALs for 1 h and then further cultured for 3 h in absence of LPS (time point corresponding to the 3-h LPS stimulation done in our microarray). Whereas C1q bound to ALs did not modulate expression of type I IFNs or IL-10 at 3 h of incubation, C1q bound to LALs significantly ($p < 0.01$) increased IL-27 mRNA levels compared with those of LALs alone (Fig. 4A), although this 2-fold increase in IL-27 was lower than the induction observed in presence of LPS (Fig. 3F).

Dying cells release numerous DAMPs that can activate TLR4, similarly to LPS (44). To model the more physiologic condition in which this release of DAMPs by EALs and LALs might affect the modulation of HMDM response by C1q, HMDMs were incubated with C1q-bound EALs or LALs for 1 h and then incubated with...
EAL or LAL conditioned media for 3 h. Although again at this time point no induction of type I IFNs or IL-10 was detectable, the presence of EAL conditioned media induced a 3-fold increase in IL-27 expression in HMDMs ingesting C1q-bound EALs compared with that after ingestion of EALs alone (Fig. 4B). Moreover, in presence of LAL conditioned media, C1q-bound LALs induced...
IL-27 levels induced by LALs alone (Fig. 5C). In addition, no change was observed in IL-10 levels after neutralization of type I IFNs (Fig. 5D), and neutralization of IL-27 slightly decreased IL-10 protein levels, but the differences did not reach statistical significance (Fig. 5E). This suggests that induction of IL-27 by C1q is partially dependent on type I IFNs in LPS-stimulated HMDMs, whereas induction of IL-10 likely involves multiple pathways.

C1q suppresses NLRP3 inflammasome activation and IL-1β cleavage

Our microarray data suggest that EALs, C1q–EALs, and C1q–LALs may decrease NLRP3 inflammasome activity through decreased NLRP3 mRNA levels and increased expression of negative regulators of inflammasome activity such as POP1/ASC2 or NLRP12 (Fig. 2 and Supplemental Table I). To test this hypothesis, PKH26-prelabeled EALs and LALs (red) with or without bound C1q were added to HMDMs at a 5:1 ratio for 1 h. HMDMs were then stimulated with LPS for 6 h with 1 mM ATP added during the last 90 min of LPS stimulation to activate the inflammasome. Cleavage of procaspase-1 was assessed by immunostaining using a green fluorescent probe specific to cleaved caspase-1, and HMDMs were stained using a blue cell tracker (Fig. 6A). About 30–40% of LPS-stimulated HMDMs and HMDMs that had ingested LALs showed cytoplasmic cleaved caspase-1 (Fig. 6A, 6B). However, HMDMs that have ingested EALs, C1q–EALs, or C1q–LALs showed almost no cleaved caspase-1 (the only cleaved caspase-1 signal detected in these HMDMs was associated with ALs themselves) (Fig. 6A, 6B). This decrease was slightly enhanced when counting only those HMDMs that had ingested C1q–LALs (3-fold decrease) versus cleaved caspase-1 in HMDMs with and without evidence of ingested C1q–LALs (2-fold decrease) (Fig. 6A, 6B). It is of note that while NLRP3 mRNA levels were downregulated in HMDMs that have ingested EALs, C1q–EALs, or C1q–LALs, the protein levels of NLRP3, as well as procaspase-1 and ASC, appear similar in all conditions at 6 h after LPS stimulation (Fig. 6C).

Next, we examined whether the inhibition of caspase-1 cleavage by C1q resulted in decreased mature IL-1β levels released by macrophages. HMDMs were incubated with C1q bound to EALs or LALs for 1 h and stimulated with LPS for 18 h with ATP added during the last 3 h. Upon LPS stimulation, pro–IL-1β levels were similarly increased in all conditions (Fig. 6D), observations in accordance with gene expression data showing similar increase in IL-1β mRNA levels in all conditions (Supplemental Table I). No mature IL-1β levels were detected in HMDM supernatants in absence of ATP (Fig. 6E). In presence of ATP, the amount of mature IL-1β released by HMDMs was significantly (p < 0.001) increased after LPS stimulation and in LPS-stimulated HMDMs that have ingested LALs compared with unstimulated HMDMs (Fig. 6E). Consistent with the observed decrease in caspase-1 cleavage, HMDMs that had ingested EALs or C1q-bound EALs or LALs showed a significant decrease (50–60%, p < 0.01) in the amount of mature IL-1β released by HMDMs (Fig. 6E). Altogether, these results demonstrate that C1q limited macrophage activation and inhibited cleavage of caspase-1 and subsequent IL-1β cleavage (Fig. 7).

Discussion

It is now well established that C1q can play a prominent role in the clearance of apoptotic cells and facilitates the rapid removal of damaged cells, thereby avoiding the release of potentially damaging intracellular components. Data presented in this study show that in addition to enhancing phagocytosis of autologous ALs, C1q...
bound to apoptotic cell “cargo” significantly influences the responses of HMDMs in a more physiologic model than our previous studies. C1q modulated several signaling pathways, increased the expression of immunoregulatory cytokines including IL-10, IL-27, IL-33, and IL-37, and inhibited NLRP3-dependent cleavage of caspase-1 and subsequent IL-1β cleavage, potentially through increased expression of negative regulators of inflammasome activity such as NLRP12 and/or POP1/ASC2 (Fig. 7).

Previous studies using apoptotic lymphocytic cell lines such as Jurkat cells have shown that C1q binds stably to late apoptotic cells but much less to early apoptotic cells (8, 45). Our data showed that C1q, at physiological concentrations, binds directly, and to the same extent, to primary human EALs and LALs, suggesting differences between primary and transformed apoptotic cells in this regard. The fact that C1q binds strongly and stably to EALs in our system supports a central role of C1q in the rapid removal of apoptotic cells to avoid autoimmunity.

C1q enhanced the uptake of EALs and LALs by HMDMs, in accordance with numerous previous reports showing an enhancement of uptake of apoptotic cells bound to C1q by different subsets of phagocytes (5, 8, 45, 46). Although previously assumed, we report that C1q remained on apoptotic cells during the phagocytosis process, suggesting that C1q can directly interact with and signal the macrophages. C1q bound to EALs and LALs indeed profoundly affected the HMDM response to LPS, this effect being sometimes dependent on the stage of the apoptotic cell (early versus late). It is likely that C1q engaged multiple receptors on HMDMs in addition to the engagement of other phagocytic receptors, such as Mer, SR-A, or CD36 (47), by the apoptotic cells themselves thus triggering a complex signaling cascade, which remains to be fully characterized, leading to the modulation of the several different pathways identified in this study. C1q bound to EALs and LALs regulated the expression of several cytokines in HMDMs. Particularly, C1q increased the expression of IL-33, a newly described member of the IL-1 family that can amplify M2 (alternative) polarization of macrophages induced by IL-13 (48, 49), which is also induced by C1q bound to LALs. In addition, C1q bound to LALs increased the expression of IL-37 (IL-1F7), a natural suppressor of innate inflammatory responses (50). In previous studies, C1q has been shown to enhance IL-10 production after LPS stimulation (8, 25, 46). Among myeloid cells, macrophages are the main source of IL-10, which is produced in response to TLR stimulation to limit and resolve inflammation (51). Recently, it has been shown that induction of IL-10 in LPS-stimulated murine macrophages results from the sequential induction of type I IFNs and IL-27 (43). Our data showed that C1q sequentially induces type I IFNs, IL-27, and IL-10 secretion in LPS-stimulated human macrophages. However, the induction of IL-27 by C1q was totally abolished after stimulation with higher dose of LPS, suggesting that the immunoregulatory effect of C1q

**FIGURE 5.** Coincubation with C1q tails or inhibition of type I IFNs reduces the induction of IL-27 by C1q in LPS-stimulated HMDMs. HMDMs were incubated with EALs, C1q–EALs, LALs, or C1q–LALs at a 5:1 ratio for 1 h (A, B) in presence of C1q tails and then stimulated with 10 ng/ml LPS for 3 h or (C–E) stimulated with LPS for 18 h in presence of 1 μg/ml control mouse IgG1 or neutralizing Abs against IFN-α and/or IFN-β (C, D) or goat IgG or neutralizing Abs against IL-27 (E). mRNA levels were determined by qRT-PCR and protein levels by ELISAs. Results represent means ± SD (n = 2 to 3 different donors, performed in duplicates), two-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.
occurs only when limited inflammation is present (i.e., during sterile inflammation). However, in presence of higher dose of LPS (i.e., higher TLR stimulation such as during an infection), the effect of C1q to dampen the inflammatory response is overwhelmed, a result that would be beneficial to promote the resolution of an infection. Moreover, addition of C1q tails inhibited in a dose-dependent manner the C1q-induced expression of type I IFNs and IL-27, consistent with the reported binding of C1q to

**FIGURE 6.** C1q decreased procaspase-1 and pro–IL-1β cleavage in LPS-stimulated HMDMs. HMDMs were incubated with PKH26-labeled EALs and LALs, preincubated with C1q, at a 5:1 ratio for 1 h and then stimulated with 10 ng/ml LPS. (A and B) HMDMs were stimulated with LPS for 6 h. ATP (1 mM) was added 90 min before the end of the stimulation. Cleaved caspase-1 was detected by FITC fluorescent caspase-1 probes, and HMDMs were stained with a blue cell tracker. Representative merged micrographs of three independent experiments (from three different donors) are shown. Scale bars, 50 μm. Areas in white boxes were enlarged to show PKH26–AL (top, red), cleaved caspase-1 (middle, green), and the merge (bottom). (B) Quantification of cleaved caspase-1 in HMDMs. (C) NLRP3, procaspase-1, ASC, and actin expression in HMDM cell extracts. Representative blots of two independent experiments are shown. (D and E) Levels of pro–IL-1β relative to β-actin levels (D, cell extracts) and mIL-1β relative to pro–IL-1β (E, supernatants) in HMDMs stimulated with LPS for 18 h with ATP added during the last 3 h of stimulation. Representative blots of three independent experiments are shown. All results represent means ± SD (n = 3 different donors), two-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.
activation, C1q may prevent excessive and dysregulated inflammasome activation induced by these DAMPs (Fig. 7). The mechanism by which C1q modulates inflammasome activation is still under investigation, but one possible hypothesis is that C1q bound to LALs increases negative regulators of inflammasome activation such as POP1/ASC2 mRNA levels. POP1 [which is not encoded in the mouse genome (53)] has been shown to bind ASC and modulate NF-κB activation and procaspase-1 cleavage (54) in human macrophages. Others have reported that inhibition of inflammasome activation by autocrine IL-10 is dependent on signaling through JAK3 (55), suggesting that C1q may induce both an early direct effect on the inflammasome (at 3–6 h through regulation of ASC2 and potentially other negative regulators) and a long-lasting effect via upregulation of type I IFNs (56), JAK3, and IL-10. Finally, C1q bound to EALs and LALs increased the expression of NLRP12, an important inhibitor of inflammatory gene expression in human myeloid cells through suppression of NF-κB activation (57). Taken together, these data indicate that more than one pathway is induced by C1q to direct macrophage polarization and inhibit inflammasome activation.

In summary, C1q enhanced phagocytosis of autologous ALs and significantly modulated gene expression profile and inflammasome activity of HMDMs (Fig. 7). Importantly, the predominant effect of C1q on HMDM inflammatory responses was observed when C1q was bound to late apoptotic cells, as early apoptotic cells themselves seem to have direct suppressive effects especially on inflammasome activation. This suggests that C1q, in the absence of other complement proteins, is a potent immunoregulatory molecule, which contributes to containing the inflammatory response induced by secondary necrosis by both immediate effects and through induction of regulatory cytokines. This study extends our initial understanding of the consequences of C1q-macrophage interactions and identifies specific and potentially novel molecular pathways induced by C1q that suppress macrophage inflammation. These results thereby identify candidate therapeutic targets to control inflammation, suppress autoimmunity (SLE), and promote host defense and/or vaccine design.
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Disclosures
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