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

*J Immunol* 2012, 188:5682-5693; Prepublished online 20 April 2012; doi: 10.4049/jimmunol.1103760

http://www.jimmunol.org/content/188/11/5682

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2012/04/20/jimmunol.1103760.DC1

**References**

This article cites 56 articles, 26 of which you can access for free at:

http://www.jimmunol.org/content/188/11/5682.full#ref-list-1

---

**Why The JI?** Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
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: 5682–5693.

The Journal of Immunology

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00.

Department of Molecular Biology and Biochemistry, Institute for Immunology, University of California, Irvine, Irvine, CA 92697

Received for publication December 22, 2011. Accepted for publication March 20, 2012.

This work was supported by Grant UL1 RR031985 from the National Center for Research Resources (a component of the National Institutes of Health and the National Institutes of Health Roadmap for Medical Research), National Institutes of Health Grants AI 41090 and AG 00538, and the Cypress College Science, Technology, Engineering, and Math Summer Bridge Program.

The gene expression data presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE30177.

Address correspondence and reprint requests to Dr. Andrea J. Tenner, Department of Molecular Biology and Biochemistry, 3205 McGaugh Hall, University of California, Irvine, Irvine, CA 92697. E-mail address: atenner@uci.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: AL, apoptotic lymphocyte; ASC, apoptosis-associated speck-like protein containing a CARD domain; DAMP, damage-associated molecular pattern; EAL, early apoptotic lymphocyte; GO, gene ontology; HMDM, human monocyte-derived macrophage; HMGB1, high mobility group box 1; HSP, heat shock protein; LAL, late apoptotic lymphocyte; PAMP, pathogen-associated molecular pattern; pDC, plasmacytoid dendritic cell; Pt, propidium iodide; qRT-PCR, quantitative real-time PCR; SLE, systemic lupus erythematosus.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00.

Department of Molecular Biology and Biochemistry, Institute for Immunology, University of California, Irvine, Irvine, CA 92697

Received for publication December 22, 2011. Accepted for publication March 20, 2012.

This work was supported by Grant UL1 RR031985 from the National Center for Research Resources (a component of the National Institutes of Health and the National Institutes of Health Roadmap for Medical Research), National Institutes of Health Grants AI 41090 and AG 00538, and the Cypress College Science, Technology, Engineering, and Math Summer Bridge Program.

The gene expression data presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE30177.

Address correspondence and reprint requests to Dr. Andrea J. Tenner, Department of Molecular Biology and Biochemistry, 3205 McGaugh Hall, University of California, Irvine, Irvine, CA 92697. E-mail address: atenner@uci.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: AL, apoptotic lymphocyte; ASC, apoptosis-associated speck-like protein containing a CARD domain; DAMP, damage-associated molecular pattern; EAL, early apoptotic lymphocyte; GO, gene ontology; HMDM, human monocyte-derived macrophage; HMGB1, high mobility group box 1; HSP, heat shock protein; LAL, late apoptotic lymphocyte; PAMP, pathogen-associated molecular pattern; pDC, plasmacytoid dendritic cell; Pt, propidium iodide; qRT-PCR, quantitative real-time PCR; SLE, systemic lupus erythematosus.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00.

Department of Molecular Biology and Biochemistry, Institute for Immunology, University of California, Irvine, Irvine, CA 92697

Received for publication December 22, 2011. Accepted for publication March 20, 2012.

This work was supported by Grant UL1 RR031985 from the National Center for Research Resources (a component of the National Institutes of Health and the National Institutes of Health Roadmap for Medical Research), National Institutes of Health Grants AI 41090 and AG 00538, and the Cypress College Science, Technology, Engineering, and Math Summer Bridge Program.

The gene expression data presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE30177.

Address correspondence and reprint requests to Dr. Andrea J. Tenner, Department of Molecular Biology and Biochemistry, 3205 McGaugh Hall, University of California, Irvine, Irvine, CA 92697. E-mail address: atenner@uci.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: AL, apoptotic lymphocyte; ASC, apoptosis-associated speck-like protein containing a CARD domain; DAMP, damage-associated molecular pattern; EAL, early apoptotic lymphocyte; GO, gene ontology; HMDM, human monocyte-derived macrophage; HMGB1, high mobility group box 1; HSP, heat shock protein; LAL, late apoptotic lymphocyte; PAMP, pathogen-associated molecular pattern; pDC, plasmacytoid dendritic cell; Pt, propidium iodide; qRT-PCR, quantitative real-time PCR; SLE, systemic lupus erythematosus.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00.

Department of Molecular Biology and Biochemistry, Institute for Immunology, University of California, Irvine, Irvine, CA 92697

Received for publication December 22, 2011. Accepted for publication March 20, 2012.

This work was supported by Grant UL1 RR031985 from the National Center for Research Resources (a component of the National Institutes of Health and the National Institutes of Health Roadmap for Medical Research), National Institutes of Health Grants AI 41090 and AG 00538, and the Cypress College Science, Technology, Engineering, and Math Summer Bridge Program.

The gene expression data presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE30177.

Address correspondence and reprint requests to Dr. Andrea J. Tenner, Department of Molecular Biology and Biochemistry, 3205 McGaugh Hall, University of California, Irvine, Irvine, CA 92697. E-mail address: atenner@uci.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: AL, apoptotic lymphocyte; ASC, apoptosis-associated speck-like protein containing a CARD domain; DAMP, damage-associated molecular pattern; EAL, early apoptotic lymphocyte; GO, gene ontology; HMDM, human monocyte-derived macrophage; HMGB1, high mobility group box 1; HSP, heat shock protein; LAL, late apoptotic lymphocyte; PAMP, pathogen-associated molecular pattern; pDC, plasmacytoid dendritic cell; Pt, propidium iodide; qRT-PCR, quantitative real-time PCR; SLE, systemic lupus erythematosus.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00.
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 Invitrogen. HL-1 medium was from BioWhittaker and defined FCS from HyClone. Recombinant human M-CSF and recombinant human IL-2 were from PeproTech. ATP was from Sigma-Aldrich. Mouse IgG1 Abs from HyClone. Recombinant human M-CSF and recombinant human IL-2 were from Invitrogen. HL-1 medium was from BioWhittaker and defined FCS RPMI 1640, 10% FCS, 2 mM L-glutamine, and 1% penicillin/streptomycin. The monocyte fraction was CD11b+. Lymphocytes were maintained for 7 d in media without FBS to generate early apoptotic lymphocytes (EALs; annexin V+/propidium iodide (PI)−). 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, Genome Center 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 I 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 Cytoscope (35). All data were submitted to the MIAME-compliant (36) 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 iQ software (Bio-Rad). The fold-change (FC) was determined as follows: FC = 2−ΔΔCt, where ΔΔCt = (CtTarget − CtGAPDH)unstimulated − (CtTarget − CtGAPDH)stimulated. Ct values were defined as the number of cycles at which the fluorescence signals were detected (38).

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 probe (ImmunoChemistry Technologies) and Cell Tracker Blue CMF-HC (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 3D2 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 >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 macromolecule 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 signalling 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-kB 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, 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

FIGURE 2. Gene expression and main biological processes modulated by C1q in HMDMs during the uptake of ALs. (A) Pearson correlation coefficient-based heat map (complete linkage method) representation of the log$_2$ fold-change (all conditions performed in triplicate) of HMDMs incubated with EALs, C1q–EALs, LALs, and C1q–LALs and then stimulated with LPS for 3 h over unstimulated HMDMs. (B) GO-based functional annotation of genes modulated by C1q in HMDMs. Major biological processes are shown as the percentage of differentially expressed annotated genes (redundancy is due to the involvement of individual genes in multiple biological processes). (C–F) Network diagrams of chemotaxis (C), inflammation/cytokines (D), JAK/STAT signaling (E), and NLRP3 inflammasome activation (F) pathways modulated by C1q in HMDMs. Node colors represent changes in gene expression in C1q–EALs versus EALs or C1q–LALs versus LALs, shown using a color gradient (blue, downregulated; white, not modulated; yellow, upregulated by C1q).
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...
a 6-fold increase in IL-27 expression in HMDMs compared with that of LALs alone (Fig. 4B), induction similar to what is observed in the presence of LPS without the conditioned media (Fig. 3F). These results suggest that C1q sequentially induced type I IFNs, IL-27, and IL-10 expression in LPS-stimulated HMDMs and may act in synergy with soluble factors released by dying cells to induce IL-27 in resting HMDMs.

**Coincubation with C1q tails or neutralization of type I IFNs suppresses IL-27 induction by C1q in LPS-stimulated HMDMs**

C1q has been shown to bind apoptotic cells through its globular heads and can interact with receptors on phagocytic cells through its tail region. Thus, to determine the specificity of C1q signaling in the induction of cytokines, HMDMs were incubated with C1q-bound EALs or LALs for 1 h in presence of increasing doses of C1q tails, which should compete with C1q bound to ALs to bind HMDMs without inducing C1q receptor clustering on HMDMs and thus no signaling. After washing to remove unbound targets and C1q tails, HMDMs were stimulated with LPS for 3 h, and mRNA levels of IFN-α and IL-27 were determined by qRT-PCR (Fig. 5A, B). When HMDMs were incubated with C1q-bound EALs or LALs in presence of C1q tails, the C1q-induced expression of IFN-α and IL-27 was significantly decreased in a dose-dependent manner by C1q tails (Fig. 5A, B), suggesting a competition between C1q tails and C1q bound to ALs to induce cytokines in HMDMs.

To determine the contribution of type I IFNs in the induction of IL-27 and IL-10 by C1q in LPS-stimulated macrophages, HMDMs were incubated with C1q-bound EALs or LALs for 1 h and then stimulated with LPS for 18 h in presence of control mouse IgG or type I IFNs or IL-27 neutralizing Abs (Fig. 5C–E). Incubation with anti–IFN-α Abs, anti–IFN-β Abs, or both significantly decreased the IL-27 secretion induced by C1q bound to EALs to levels of EALs alone (Fig. 5C). The induction of IL-27 in HMDMs by C1q bound to LALs was also significantly decreased by anti–IFN-α Abs and/or anti–IFN-β Abs, however the levels of IL-27 after neutralization of type I IFNs remained significantly higher than 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 30 min of LPS stimulation to activate the inflammasome. Cleavage of pro-caspase-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 pro-caspase-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.
Main biological pathways modulated by C1q in HMDMs. C1q increases the expression of type I IFNs and IL-27, known to act sequentially to stimulate expression of IL-10 (also upregulated by C1q); IL-33, known to promote alternative activation of macrophages; IL-37, a potent natural suppressor of innate inflammatory responses; and JAK3, which may be involved in IL-27 upregulation. C1q also suppresses pro-caspase-1 and pro–IL-1β cleavage and subsequent mIL-1β release through possibly increased expression of negative regulators of inflammasome activity and indirectly (at later times) through increased IL-10 expression, which is known to decrease pro–IL-1β mRNA levels. C1q may thus prevent excessive and dysregulated inflammasome activation induced by the release of DAMPs (HMGB1, HSPs, ATP) from apoptotic cells during secondary necrosis that can activate TLR4 and the NLRP3 inflammasome. Orange arrows indicate genes upregulated by C1q, and blue lines indicate genes downregulated by C1q.

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.
Disclosures

The authors have no financial conflicts of interest.

References


