UNIVERSITY OF CALIFORNIA,
IRVINE

Complement Protein C1q: an Immunological Rheostat That Sculpts the Innate and Adaptive Immune Response During the Phagocytosis of Dying Cells

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Elizabeth Victoria Clarke

Dissertation Committee:
Professor Andrea J. Tenner, Chair
Professor Craig M. Walsh
Professor Melissa B. Lodoen

2014

DEDICATION

To

My husband Stephen Knox: my best friend, my climbing partner and the love of my life;

My loving and supportive family: parents Allan and Colette Clarke, sisters Deborah and Julia Clarke, grandparents Paul and Sabine Ruthman and Faye Clarke; family in-law: Mary-Grace, Liz, John, and David Knox, Lois and Jack Caldwell, Ruth Barrett, Pat and Jim Bennett

My dear friends Leslie Glustrom, Julie Blair, Jocelyn Woods, Erika Kubik, Sara Symons, Sarah Nadeau, Lisa and Denis Geoghegan, Stacey Wagner, Dan Cash, Lanny Gov, Michael Hernandez, Heather Straub, Paul and Mary Ernst, Diane Getty, Celso Espinoza, Max Obermiller, Grace Sim, Jessica Dodson, Kate Ball, John Palisano, Karen McGlothlin, and Jennifer Michael;

My wonderful medical team including Michael Prados, Susan Knight, Mitchel Berger, Carol Hathaway-Clark and Fred Hochberg

Thank you for your continued love, encouragement and wisdom and for helping me to get back up, continue the race and achieve my dreams.

"These were wondrous days, with moments of incredible beauty, hours of fatigue and seconds of panic."
Charles Houston, on climbing K2, 1939

"Fail Better."
Samuel Beckett, *Worstward Ho*, 1983

"The real voyage of discovery consists not in seeking new landscapes but in having new eyes."
Marcel Proust, *In Search of Lost Time*, 1923

"Our doubts are traitors, and make us lose the good we oft might win by fearing to attempt."
William Shakespeare, *Measure for Measure*, Act I, Scene IV, 1604

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

APC, antigen-presenting cell; APC, antigen-presenting cell; ASC, apoptosis-associated speck-like protein; DAMPs, damaged associated molecular patterns; DC, dendritic cell; EAL, early apoptotic lymphocytes (EAL); HMGB1, high mobility group box 1; iDC, immature dendritic cell; LAL, late apoptotic lymphocyte; LPS, lipopolysaccharide; Mϕ, human monocyte-derived macrophages; NLRP, NLR family pyrin domain containing; PAMPs, pathogen associated molecular patterns; pDC, plasmacytoid dendritic cell; PD-L1, programmed-death ligand 1; PD-L2, programmed death ligand 2; POP1, PAAD-only protein-1; PBMCs, peripheral blood mononuclear cells; rhGM-CSF, recombinant human granulocyte macrophage colony-stimulating factor; rhM-CSF, recombinant macrophage colony- stimulating factor; SLE, systemic lupus erythematous; Teff, T effector cell (Th1, Th17 or CD8+IFN-γ+); Treg, regulatory T cell (CD4+Foxp3+ or CD8+Foxp3+).

ACKNOWLEDGMENTS

I would like to express the deepest appreciation to my thesis advisor, Professor Andrea J. Tenner, an outstanding mentor and scientist. She is exceptionally dedicated to her trainees' scientific and intellectual development and I am extremely grateful for all she has taught me. For the past four years, she has trained me to be a careful and thoughtful researcher and has inspired me to approach my study of immunology with wonder, enthusiasm and rigor. Without her guidance and continual help this dissertation would not have been possible.

I would also like to thank my committee members, Professor Craig M. Walsh and Professor Melissa B. Lodoen for their thoughtful comments, guidance and creative ideas throughout the course of my thesis work.

In addition, I thank Professor Edward L. Nelson and Professor David A. Fruman for critical review and insightful discussion of my data, and Professor Aimee L. Edinger for generous availability of her LSRII instrument.

I also thank Dr. Deborah A. Fraser and Dr. Marie E. Benoit for generously taking the time to patiently train me as a new immunologist. They are wonderful teachers and creative, thoughtful scientists. I am very lucky to have worked with both of them and benefited from their considerable expertise.

I thank the Journal of Immunology and Journal of Leukocyte Biology for permission to include Chapters 2 and 3, respectively, as part of my thesis/dissertation as these chapters were originally published in these respective journals. Chapter 2 contains data that was collected by or under the direction of Dr. Marie E. Benoit. In addition, portions of the Introduction are extracted from a review article, which Dr. Andrea J. Tenner and I published in the Journal of Leukocyte Biology.

These studies were supported in part by NIH AI-41090, T32 AI 60573 and the Lupus Foundation of America G. M. Finzi fellowship. In addition, acquisition of human blood was supported by the National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health, through Grant UL1 TR000153. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

CURRICULUM VITAE

Elizabeth Victoria Clarke

Education

University of California, Irvine—PhD Candidate, department of Molecular Biology and Biochemistry (9/2009 – 9/2014)

- Thesis project: discovered a novel regulatory role for complement protein C1q in modulating macrophage and dendritic cell-mediated T cell activation, pinpointing targets for preventative intervention in autoimmunity

Boston University—MPH, Environmental Health (2007 – 2009)

- Research: demonstrated pure antagonism with two novel chemical antagonists of the constitutively active Aryl Hydrocarbon Receptor (AhR) in breast cancer cells

University of Colorado—MS, Chemistry (2003 – 2005)

- Research: Nuclear Magnetic Resonance (NMR) studies on the human Estrogen Receptor (ERβ) bound to several toxic environmental ligands

University of the South—BS, Biology/Biochemistry (1999 – 2003)

Publications

- **Clarke, E. V.**, Weist, B. M., Walsh, C. M., and Tenner, A. J. The association of complement protein C1q with apoptotic cells ingested by human macrophages modulates T cell subset proliferation and induction. Journal of Leukocyte Biology. *In Press* (2014).
- **Clarke, E. V.** and A. J. Tenner. 2014. Complement modulation of T cell immune responses during homeostasis and disease. Journal of Leukocyte Biology. 2014 Sep 10. pii: jlb.3MR0214-109R. [Epub ahead of print]
- **Clarke, E. V.**, Benoit, M. E., & Tenner, A. J. Purification of Human Monocytes and Lymphocyte Populations by Counter Current Elutriation: A Short Protocol. Bio-Protocols (2013).http://www.bio-protocol.org/wenzhang.aspx?id=981#!
- Ueno, N., Harker, K. S., **Clarke, E. V.**, McWhorter, F. Y., Liu, W.F., Tenner, A. J. and Lodoen, M. B. Real-time dynamic imaging reveals Mac-1-mediated transmigration of *Toxoplasma gondii*-infected human monocytes across endothelial barriers. 2014 Apr;16(4):580-95. doi:10.1111/cmi.12239. Epub 2013 Dec 4.
- Benoit, M. E., **Clarke, E. V.**& Tenner, A. J. C1q binding to and uptake of apoptotic lymphocytes by human monocyte-derived macrophages. Bio-Protocols (2013). http://www.bio-protocol.org/wenzhang.aspx?id=877.
- Benoit, M. E., **Clarke, E. V.**, Morgado, P., Fraser, D. A. & Tenner, A. J. Complement protein C1q directs macrophage polarization and limits inflammasome activity during the uptake of apoptotic cells. *J. Immunol.* 188, 5682–5693 (2012).

Abstracts

- **E. V. Clarke** and A. J. Tenner. The association of complement protein C1q with apoptotic cells ingested by human dendritic cells modulates dendritic cell-mediated T cell activation. Presented at the Annual Meeting of the American Association of Immunologists (May 2014, Pittsburgh, PA)
- **E. V. Clarke**, C. M. Walsh and A. J. Tenner. Complement protein C1q modulates macrophage-mediated T cell activation during the phagocytosis of dying cells. Selected for an oral presentation at the annual Society for Leukocyte Biology meeting (October 2013, Newport, RI).
- **E. V. Clarke**, B. M. Weist, C. M. Walsh and A. J. Tenner. Complement protein C1q influences macrophage-mediated T cell activation. Selected for an oral presentation at the Annual Meeting

of the American Association of Immunologists (May 2013, Honolulu, HI)

- **E. V. Clarke**, D. A. Fraser, M. E. Benoit and A. J. Tenner. C1q activates distinct signaling mechanisms in human monocytes, macrophages and immature dendritic cells. Presented at the Annual Meeting of the Society for Leukocyte Biology (September 2011, Kansas City, MO).

Honors and Awards

- American Association of Immunologists Trainee Poster Award for the annual meeting (2014)
- American Association of Immunologists Trainee Abstract Award for the annual meeting (2013)
- Selected oral presentation at the annual Society for Leukocyte Biology (2013)
- Selected oral presentation at the annual American Association of Immunologists meeting (2013)
- UC Irvine NIH Immunology T32 Training Grant (2013)
- First Prize Poster Award at the UC Irvine Institute for Immunology Fair (2012)
- Lupus Foundation of America 2012 G. M. Finzi Memorial Student Fellowship (2012)
- Society for Leukocyte Biology (SLB) Travel Award towards 2011 Annual Meeting
- Boston University School of Public Health Scholarship (2007)
- Molecular Pharmacology NIH training grant (2004)
- Undergraduate Dean's List (2000 – 2003)

Professional Societies

- Union of Concerned Scientists (since 2014)
- American Association of Immunologists (since 2013)
- American Public Health Association (since 2013)
- Society for Leukocyte Biology (since 2011)
- Association for Women in Science (since 2010)
- American Association for the Advancement of Science (since 2010)
- Society of Toxicology (since 2008)

Teaching Experience

- Teaching Assistant for undergraduate AIDS Fundamentals (2013)
- Teaching Assistant for undergraduate Immunology (2011, 2013)
- Teaching Assistant for undergraduate Microbiology laboratory (2011)
- Teaching Assistant for undergraduate Genetics (2011)
- Chemistry tutor (2005)
- Teaching Assistant for undergraduate General Chemistry (2003 – 2004)

Students Supervised

- Jaydee Choompoo, 2011 – 2012
- Ania Alam, 2012
- Keng-Tsai Lin, 2013
- Vy Nguyen, 2014

Technical and Language Skills

- Proficiency with SigmaPlot, Prism, FlowJo, FlowLogic, Adobe Photoshop and Adobe Illustrator, Microsoft Excel, Word, PowerPoint, software
- Experience with Stata and R statistical analysis software
- Developed a novel mixed-leukocyte reaction (MLR) with primary human cells for evaluating the role of complement protein C1q on macrophage-mediated T cell activation
- Developed a novel ELISA for evaluating relative binding differences in purified human protein samples containing Alzheimer's Disease-related SNPs

- Proficiency with FACS analysis including 7-color flow cytometry for T cell lineage analysis
- Trained in surface plasmon resonance (SPR) for evaluating protein-protein binding affinity
- Proficiency with phosphoprotein analysis
- Proficiency with purification of human peripheral leukocytes, counter-current elutriation of primary human monocytes, and T cell subset isolation
- Proficiency with primary human cell culture (monocytes, macrophages, lymphocytes)
- Proficiency with C1q purification from human plasma
- Proficiency with SDS-PAGE and western blotting
- Proficiency with light microscopy
- Conversational knowledge of French

Work Experience

Biogen-Idec, Stability Operations Associate, Cambridge, MA, 2007

- Management of clinical research studies and pharmaceutical products

Boston University, Research Associate, Boston, MA, 2006

- Studies on bacterial gene regulatory networks that eliminate environmental toxic heavy metals

ABSTRACT OF THE DISSERTATION

Complement Protein C1q: an Immunological Rheostat That Sculpts the Innate and Adaptive Immune Response During the Phagocytosis of Dying Cells

By

Elizabeth Victoria Clarke

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2014

Professor Andrea J. Tenner, Chair

Deficiency in C1q, the recognition component of the classical complement cascade and a key opsonin involved in apoptotic cell clearance, leads to lupus-like autoimmune diseases characterized by auto-antibodies to self proteins and aberrant T and B cell activation. Studies have suggested that these pathological consequences may result from impaired clearance of apoptotic cells. To investigate how C1q may modulate inflammation resulting from diminished apoptotic cell clearance, I applied a novel system consisting entirely of primary human cells: human monocyte-derived macrophages (Mϕ) and dendritic cells (DC), ingesting autologous apoptotic human lymphocytes (C1q-polarized Mϕ or C1q-polarized DC), and either autologous or allogeneic human T cells. This physiologically relevant experimental system enabled characterization of the C1q-polarized Mϕ or C1q-polarized DC functional phenotype and subsequent Mϕ and DC-mediated T cell activation, avoiding the caveats of other systems using either transformed cell lines or non-physiologic presentation of C1q. The results demonstrate that C1q influences intracellular signaling and gene expression of both secreted protein and cell surface receptors. That is, C1q-polarized Mϕ exhibited enhanced STAT1 phosphorylation that is

correlated with attenuated NLRP3 inflammasome activation and sequential induction of type I IFN-s, IL-27, and IL-10 in LPS-stimulated Mϕ relative to Mϕ ingesting apoptotic lymphocytes alone. C1q-polarized DC also exhibited enhanced IL-27 expression relative to DC ingesting apoptotic lymphocytes without C1q. Under the same conditions C1q-polarized Mϕ showed suppressed induction of CD40 and enhanced expression of PD-L1 and PD-L2 expression while C1q-polarized DC exhibited reduced CD86 induction and elevated PD-L2 and CD39 expression relative to DC ingesting apoptotic cells alone. Finally, C1q-polarized Mϕ reduced allogeneic and autologous Th17 and Th1 subset proliferation, and initiated a trend towards increased regulatory T cell proliferation relative to Mϕ ingesting LAL alone. Moreover, relative to DC ingesting AC in the absence of C1q, C1q-polarized DC decreased autologous Th17 and Th1 proliferation. These data demonstrate that a functional consequence of C1q-polarized Mϕ and DC is the regulation of T effector cell activation, thereby "sculpting" the adaptive immune system to avoid autoimmunity while clearing dying cells. Importantly, these studies identify novel target pathways for therapeutic intervention in SLE and other inflammatory autoimmune diseases.

Chapter 1 — Introduction

C1q: beyond the classical complement pathway

The complement system is a powerful immune system mechanism. There are over 30 proteins comprising this system and all can be present in both circulation and in extracellular fluid upon injury. Upon activation, a series of proteolytic and protein-protein interactions occur, resulting in the opsonization of invading pathogens or dangerous material, the recruitment of leukocytes to the site of infection or injury, and the lysis of the pathogen (Ricklin and Lambris, 2013). Three complement system activation pathways have been observed: the classical pathway, alternative pathway and lectin pathway (**Figure 1.1**) (Bohlson et al., 2007).

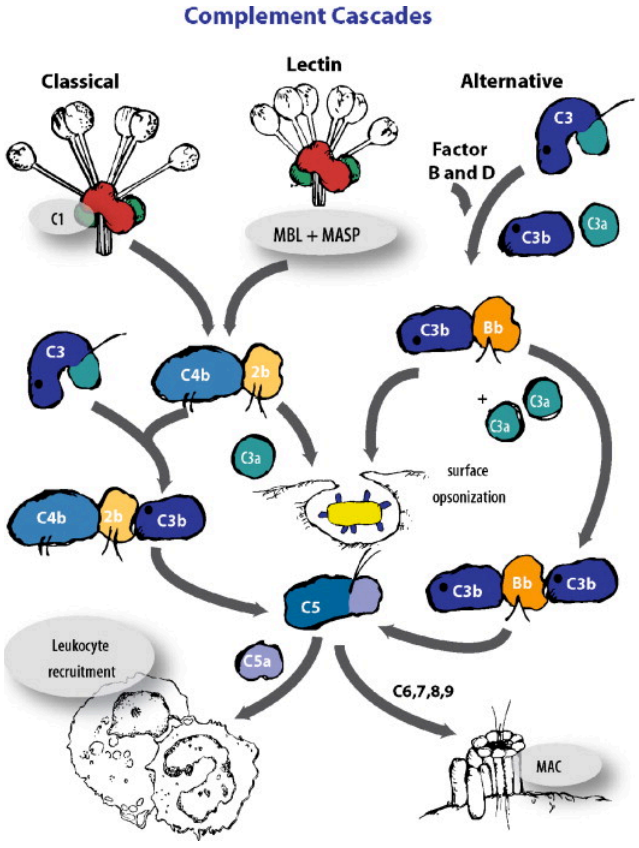


Figure 1.1 – Schematic diagram of complement activation. The products that result from activation of either the classical, lectin or alternative pathway, and the effector functions of surface opsonization (C3b deposits on the pathogen (yellow) and is engulfed by a phagocyte), leukocyte recruitment (C3a and C5a), and pathogen lysis by formation and insertion of C5b-9 complex in membranes (MAC, membrane attack complex) are depicted. (Adapted from (Bohlson et al., 2007)).

Complement protein C1q is the pattern recognition component in the classical pathway activation of complement (Ghai et al., 2007). Circulating C1q is normally present in complex with C1r and C1s as the macromolecular initiator of the classical complement pathway, C1. However, upon conversion of the C1r and C1s proenzymes to active serine protease as a result of C1 binding to an activator, the C1-inhibitor protein irreversibly binds to and inactivates C1r and C1s proteases, dissociating them from the C1 complex, and this enables C1q to function independently of the rest of the C1 complex (Gompels et al., 2005). In addition, C1q can be synthesized in the absence of other complement components by myeloid cells *in vitro* (Benoit et al., 2013;Bensa et al., 1983) and is upregulated in the brain in response to injury, as reviewed in (Veerhuis et al., 2011). Moreover, lower serum levels of C1q and lower peritoneal macrophage production of C1q is associated with a polymorphism in the C1q gene regulatory region that has been linked to murine lupus nephritis and impaired in vivo clearance of apoptotic cells (reviewed in (Sontheimer et al., 2005)). In addition, humans with a genetic deficiency in C1q present with systemic lupus erythematous (SLE) with nearly 100% penetrance (Walport et al., 1998). These data are consistent with a key function for C1q in limiting inflammation and/or promoting repair.

Systemic Lupus Erythematous

SLE is a systemic, chronic inflammatory autoimmune disease characterized by anti-nuclear (and other) autoantibody production by hyper-stimulated B cells, Th17 and Th1 cell over-activation, reduced Treg, and attenuated phagocytosis (Tsokos, 2011;Bijl et al., 2006). Patients present with clinical features such as skin rashes, hair loss, arthritis, serositis, kidney and even brain / CNS damage, among others (Tsokos, 2011). Approximately 90% of the individuals

diagnosed with SLE are women (Harley et al., 2008), perhaps due in part to estrogen-dependent AID activation (Feng et al., 2006). In addition, the incidence and prevalence of SLE is higher among non-white racial groups (Tsokos, 2011).

Presently, the mechanism for how SLE is initiated is not fully understood. However, one contributing factor may be a defect in clearing apoptotic cells before they undergo secondary necrosis, as this would result in the release of inflammatory contents of these cells (reviewed in (Elkon and Santer, 2012)). Such inflammation would also trigger APC-mediated T cell activation, ultimately leading to B cell stimulation and autoantibody production (Botto et al., 1998). Given that humans deficient in C1q develop SLE at some point during their lifetime with close to 100% penetrance, it is hypothesized that C1q is likely contributing to the suppression of autoimmunity, particularly in this initiation phase of the disease (reviewed in (Fraser and Tenner, 2008)). Thus, the objective of this thesis is to examine the mechanism of how C1q may be avoiding autoimmunity by characterizing the function of C1q on (a) APC functional phenotype and (b) on APC-mediated T cell activation during the phagocytosis of dying cells. The data I present here demonstrate a clear role for C1q in attenuating APC and T cell inflammation in this context, suggesting one mechanism by which C1q may contribute to avoiding autoimmunity.

A role for C1q in enhancing phagocytosis and resolving inflammation

C1q triggers enhanced phagocytic activity when presented to the phagocyte in a multivalent fashion such as when immobilized on a surface or bound to a particle such as apoptotic cells (AC) (reviewed in (Fraser and Tenner, 2008)). Our lab has previously demonstrated that C1q increases phagocyte ingestion of AC and increases anti-inflammatory

cytokines such as IL-10 (Fraser et al., 2009) to limit inflammation (Fraser et al., 2006;Fraser et al., 2007;Fraser et al., 2009). Indeed, earlier studies in the Tenner Lab showed that primary human monocyte-derived dendritic cells (DCs) ingesting C1q-bound apoptotic cells are polarized to an anti-inflammatory state relative to DCs ingesting apoptotic cells alone, as evidenced by elevated IL-10 production and diminished CCL3 production (Fraser et al., 2009). Other labs have demonstrated that C1q has an anti-inflammatory effect on DC phenotype. For example, Roos and colleagues found that C1q enhances the uptake of apoptotic cells by DCs (and Mϕ) in a dose-dependent (0 – 60 μg/ml) manner, resulting in a higher production IL-10 (Nauta et al., 2004). Additionally, the same group found that differentiation of primary human DC in the presence of C1q causes the resulting DC to have elevated phagocytic capacity and diminished expression of CD80, CD83 and CD86 (Castellano et al., 2007).

Moreover, Lu and colleagues showed that primary human monocyte-derived DCs interacting with immobilized C1q during their differentiation from monocytes have elevated IL-10 and decreased IL-23, TNF-α and IL-12 as well as enhanced ERK, p38 and PI3K/p70S6K signaling and decrease the percentages of Th1 and Th17 proliferation in an allogeneic MLR in the presence of exogenous IFN-γ (0.1 μg/ml) (Teh et al., 2011). Finally, Elkon and colleagues discovered that plasmacytoid DCs interacting with C1q-containing immune complexes exhibited a substantial reduction in IFN--response genes (Santer et al., 2012). These observations were corroborated by data demonstrating that in the absence of C1q, immune complexes quickly trafficked to lysosomes for degradation, however in the presence of C1q, immune complexes remained in early endosomes for at least 4 hours. Moreover, C1q-deficient patients had elevated serum and cerebrospinal fluid levels of IFN-α and IFN-γ-inducible protein-10 levels, which were strongly correlated with higher autoantibody titers. Collectively these data highlight a role for

C1q in preventing the development of immune complex-stimulated autoimmunity (Santer et al., 2012).

Thus, C1q acting on Mϕ and DC, presumably in the tissue functions like a rheostat or biosensor (Figure 1.2) (Bohlson et al., 2007), polarizing the antigen-presenting cell (APC) towards an anti-inflammatory state. Whether the C1q-induced anti-inflammatory effect observed in human Mϕ and DC influences the adaptive immune system during the phagocytosis of dying cells – a physiologically relevant context to the initiation and propagation phases of SLE – has not yet been explored. Given that one cause of SLE stems from deficiency in T cell peripheral tolerance, it is reasonable to propose that C1q may facilitate tolerance by attenuating inflammatory T cell effector function. I will discuss the rationale for this hypothesis further with respect to complement modulation of T cells below.

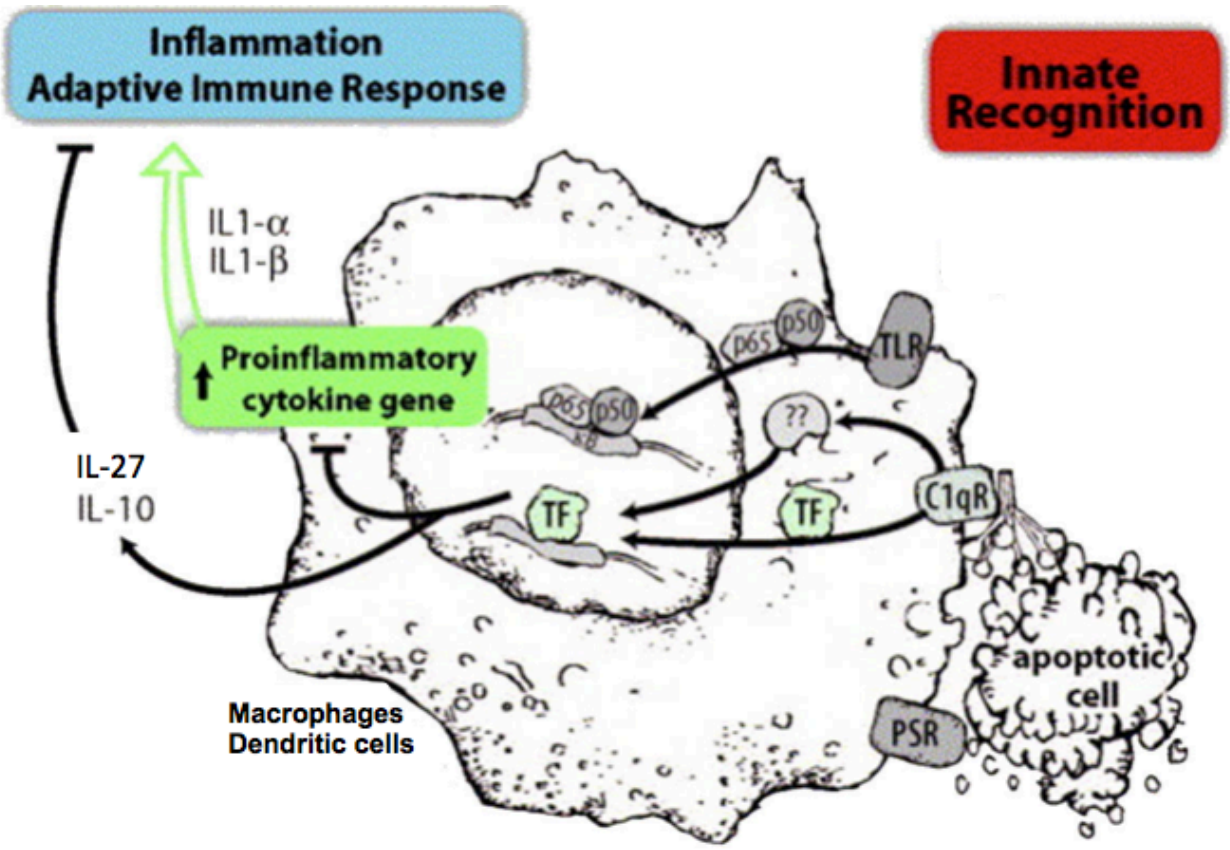


Figure 1.2 – Proposed model of phagocytic cells as a "biosensor." Mϕ and DC are antigen processing and presenting cells that functionally integrate the signals received from the particles being detected via their diverse pattern recognition receptors to initiate a gene expression program that generates new signals (cytokines), potentially to direct the subsequent adaptive immune response. (TF, transcription factor; TLR, Toll like receptor; PSR, phosphatidyl serine receptor; C1qR, C1q receptor.) (Adapted from(Bohlson et al., 2007)).

Macrophage and Dendritic Cell Functional Polarization

For the past 20 years, macrophages have generally been classified as "M1" or "classically activated" and "M2" or "alternatively activated." M1 are pro-inflammatory / highly activated, producing IL-12, IL-23, IL-1α, IL-1β and TNF-α, promoting the Th1 and Th17 T cell lineages, and killing intracellular pathogens (Lech et al., 2012;Krausgruber et al., 2011). "M2" are anti-inflammatory, producing IL-10, TGF-β, IL-4 and IL-13, promoting the Treg and Th2 T cell lineages and (depending on the subtype) killing extracellular parasites or promoting

immunoregulation (Lech et al., 2012). The M2 macrophage population more recently has been further subdivided into M2a, M2b and M2c. M2a or "alternatively activated" macrophages are induced by IL-4 or IL-13 and are involved Th2 responses, allergy and external parasite killing. M2b or "type II activated macrophages" are activated by immune complexes in combination with IL-1β or LPS and promote immune regulation and Th2 responses (Lech et al., 2012;Biswas and Mantovani, 2010). The M2c "suppressor / anti-inflammatory" macrophages can result from stimulation with IL-10, TGF-β, or glucocorticoids (Martinez et al., 2008) or the uptake of apoptotic cells (Anders and Ryu, 2011). This macrophage subpopulation is characterized by the down-regulation of pro-inflammatory cytokines, increased scavenging activity and a functional role in promoting epithelial and vascular repair via angiogenesis (Lech et al., 2012;Jetten et al., 2014;Biswas and Mantovani, 2010). Given that the Tenner lab and others have found that C1q polarizes macrophages towards an anti-inflammatory function (reviewed in (Elkon and Santer, 2012)), macrophages interacting with C1q readily ingest apoptotic cells (Zizzo et al., 2012), and recently C1q has been discovered to be heavily deposited on newly formed vessels in granulation tissue and shown to promote angiogenesis and wound healing in both mouse and rat experimental models (Bossi et al., 2014), I posit that macrophages ingesting C1q-coated apoptotic cells are M2c or M2c-like macrophages.

Dendritic cells have also been classified into specific subsets relating to their phenotype and function. Specifically, four DC subsets have been identified in the human spleen that resemble those found in human blood, which generally fall into either CD1/c+(classical), CD141+, CD16+ or CD304+ (plasmacytoid) DC subsets(Zizzo et al., 2012). CD141+ DC s have higher levels of TLR3 than other DC subsets, TLR9 is expressed exclusively by human plasmacytoid DC, neither TLR4 nor TLR7 are expressed by CD141+ or CD16+ DC subsets and

CD1b/c+ DC express only low levels of TLR7 compared with CD304+ (or CD123+) plasmacytoid DC (Mittag et al., 2011). Thus, human DC subsets have distinct, specialized roles for recognizing specific pathogen groups (Mittag et al., 2011). In addition, CD304+ plasmacytoid DC are poor stimulators of allogeneic CD4+ T cell proliferation, whereas CD1b/c+ DC stimulate CD4+ T cells most strongly followed by CD141+ DC and CD16+ DC (Mittag et al., 2011). A recent study corroborated these data, demonstrating that CD1c+, CD141+ and CD16+ myeloid DCs and CD304+ or CD123+ plasmacytoid DCs express distinct types and levels of pattern recognition receptors (PRRs) (Lundberg et al., 2014). In the present studies, I will focus on classical (myeloid) DCs, which reside in tissue where they ingest apoptotic cells and sample their environment. Upon activation by PAMPS/DAMPS, they migrate to local lymph nodes, where they present processed antigen to T cells and produce cytokines and other immunoregulators to influence the subsequent adaptive response (Lech et al., 2012). Generally, classical DCs are unique in several ways. Under homeostatic conditions, they are localized to nonlymphoid tissues and the spleen marginal zone, where they continually acquire peripheral antigens. Additionally, they are extremely adept at antigen processing and presentation, and once loaded with antigens, they have a superior migratory ability to migrate to the T cell zone of lymph nodes during homeostasis and inflammation. Finally, classical DCs are highly efficient at priming naïve T cell responses (Merad et al., 2013).

T cell subsets

Lymphocytes are derived from the lymphoid lineage and include T cells, B cells and natural killer (NK) cells. T cells can be further divided into CD4+ ("helper" which are activated by extracellular antigen presented on MHC II), and CD8+ ("cytotoxic" which are activated by

intracellular antigen presented on MHC I) T cells (Maecker et al., 2012;Maecker et al., 2012). Broadly, both CD4+ and CD8+ T cells can either be classified as naïve (CD45RA+CD45RO-) or memory (CD45RO+CD45RA-) cells (Sallusto et al., 2004). Memory T cells are subdivided into central memory T cells (T_{CM}), which are present in secondary lymphoid tissue and express CCR7 and CD62L (L-selectin), produce IL-2 and proliferate substantially in response to antigen presentation and effector memory T cells (T_{EM}), which are present in the periphery and lack CCR7 and CD62L expression, produce cytokines such as interferon IFN-γ that stimulate macrophages and other leukocytes and are less proliferative. Recent studies have shown that some memory T cell populations permanently remain in peripheral tissues after an infection is cleared (so-called tissue-resident memory T cells, T_{RM}) (reviewed in (Bretschneider et al., 2013)).

Beyond these general classifications, CD4+ T helper (Th) cells are further divided up into several main subsets or lineages according to their specialized function and phenotype. Recently, these subsets have been shown to exhibit plasticity, but in general, Th1 cells produce IFN-γ via the T-bet transcription factor and are effective against intracellular pathogens. Th2 cells produce IL-4 and IL-13 via Gata3, and Th17 cells produce IL-17 via Rorγt and exhibit effector activity against parasites. Treg cells produce IL-10, IL-35 and TGF-β via Foxp3 in order to suppress the immune responses of other immune cells. T follicular helper cells (Tfh) produce IL-21 via Bcl-6 (O'Shea and Paul, 2010). Whereas Th1, Th2, Th17 and Treg cells circulate throughout the periphery and secondary lymphoid tissue, Tfh cells function specifically in the secondary lymphoid tissue to help B cells make antibodies to T cell dependent antigens (Sathaliyawala et al., 2013). Interestingly, IL-17 released by Th17 cells is required for Tfh retention in the germinal center to stimulate autoantibody-producing B cells (Ding et al., 2013), demonstrating

one way that Th17 cells promote autoimmunity. On the other hand, in mice, a CD8+ regulatory T cell subset was recently discovered to be critical for maintenance of self-tolerance and prevention of lethal systemic-lupus-erythematous-like autoimmune disease via interaction with Tfh cells resulting in subsequent suppression of antibody (including autoantibody) production (Kim et al., 2010).

Complement modulation of T cell function

Long regarded as functioning strictly in the innate immune system, the complement system has in the last few decades been shown to have substantial contributions to directing or "sculpting" adaptive immunity, including T cell function. In the past, complement was thought to function largely as part of the innate immune system via pattern recognition receptors, anaphylatoxin-mediated recruitment of leukocytes or lysis of the pathogen. The classical complement pathway has also long been known to bind and be activated by antibodies bound to their specific pathogenic antigens, resulting in killing of the bound pathogen by complement-mediated lysis or enhancing phagocyte-mediated ingestion and/or killing. However, a rapidly growing body of literature has reported novel functions for complement proteins in modulating adaptive immunity, including both direct and indirect effects on T cell activation. In particular, reports from several groups have highlighted roles for complement component fragments C3b, iC3b, C3a and C5a and complement regulators CD46 (MCP) and DAF (CD55) as modulators of T cell activation. In addition, a few reports have suggested that C1q may influence T cell activation but these studies have a number of caveats and the role of C1q remains to be defined in this context.

Overall, complement has been shown to modulate T cell function during both homeostasis and disease in humans and in mice, though there are some significant differences in complement mediated T cell responses between murine models and human physiology as will be discussed below.

Complement component C3

While the initiation of the complement cascade can occur via three distinct pathways, the classical, lectin and alternative pathways, all three activation pathways result in the formation of a C3-convertase (C4b2b or C3bBb) that proteolytically cleaves complement protein C3 into C3a and C3b (Ricklin et al., 2010). C3a is a potent anaphylatoxin involved in the recruitment of leukocytes to the site of infection or injury, while C3b as part of both C3 and C5 convertases contributes to the continuation of the complement cascade and pathogen lysis and as an opsonin covalently linked to the activator that can induce ingestion by the phagocyte (reviewed in (Ricklin et al., 2010)). Additionally, Factor I cleaves C3b to iC3b and subsequently to C3d, the latter of which enhances B cell responses to antigen ((Carter and Fearon, 1992) and reviewed in (Carroll and Isenman, 2012)). Inherited complement C3 deficiency was first described over 40 years ago (Alper et al., 1972) and results in recurrent pyogenic infections and immune-complex disorders such as glomerulonephritis (Singer et al., 1994). Subsequent studies reporting a C3 contribution to germinal center formation and antibody responses followed and have been validated with intermittent reports over a 40-year period; these reports have been recently reviewed elsewhere (Carroll and Isenman, 2012).

Decades of data have underscored a function for C3 fragments as a modulator of T cell proliferation and differentiation either directly (by interacting with receptors on the T cell) or

indirectly (by interacting with receptors on an APC, which then influences T cell activation). Exploring possible functions of C3 in T cell biology, Strainic and colleagues showed in a murine system that after 1 hour co-culture with OVA peptide, C3aR and C5aR were upregulated in mouse OT-II T cell receptor (TCR) transgenic cells and dendritic cells (DCs) in an antigen (OVA)-dependent fashion (Strainic et al., 2008). Costimulatory signaling through CD28 on the T cell also induced C3aR and C5aR expression along with low levels of C3, factor B, factor D and C5 (Strainic et al., 2008). Although these experiments did not show the specificity of the antibodies to C3aR and C5aR by using cells from receptor knockout, $C3aR^{-/-}$ or $C5aR^{-/-,}$ animals, functional studies using APC and T cells from $C3aR^{-/-}C5aR^{-/-}$ animals and pharmacologic blockade of the two receptors did prevent the upregulation of IFN-γ (by ~75%) upon antigen stimulation by APC, providing support for a positive contribution of the signaling of these receptors in the quantitative induction of Th1 T cells.

The same group recently demonstrated that an absence of C3aR and C5aR signaling on murine CD4+ T cells stimulated with anti CD3, CD28 plus IL-2 resulted in enhanced production and secretion of IL-10, elevated TGF-β expression, and corresponding induction of Foxp3+ regulatory T cells (iTreg) with robust suppressive activity. Chemical antagonism as well as antibody blockade of C3aR andC5aR was shown to induce functional human iTregs from naive T cells incubated with autologous DCs, anti-CD3, and IL-2 (this was not observed with added TGF-β alone if C3aR and C5aR signaling was intact)(Strainic et al., 2013). These data were supported by similar experimental results in human systems by Heeger and colleagues (van der Touw et al., 2013), and subsequently extended to natural Treg (nTreg) in mice both *in vitro* and *in vivo* (Kwan et al., 2013). That is, the absence of murine C3aR and C5aR signaling in thymus-derived CD4+Foxp3+ natural Treg cells (nTreg) (during T cell stimulation) resulted in elevated

nTreg cell Foxp3 expression and promotion of nTreg cell function (Kwan et al., 2013). The C3a and C5a signaling pathways in all cells include activation of PI3Kγ and subsequently Akt (Kwan et al., 2013;Strainic et al., 2013;Strainic et al., 2008) which while promoting Th1 activation, in Treg cells results in upregulation of pFOXO1 (preventing Foxp3 induction (Kwan et al., 2013;van der Touw et al., 2013)) and thus preventing induction of suppressive activity—all positive activities for a host under invasion by a pathogen.

Other *in vivo* studies also support a role for C3 in diminishing regulatory cell function. That is, a lack of C3 correlates with Treg expansion and compromised T cell immunity in murine and human abdominal sepsis (Yuan et al., 2013;Yuan et al., 2012) and C3 deficiency expands regulatory T cells to prevent induced autoimmune diabetes in a murine model (Gao et al., 2011). None of these *in vivo* reports pinpointed whether C3a (or C3b or potentially subsequently generated C5a) was acting directly on the T cell or via an indirect APC-mediated mechanism. However, the observations are consistent with the idea that an of absence of C3aR and C5aR on the T cells promotes regulatory activity.

Several studies investigating the expression of the C3a receptor (C3aR) failed to detect it on primary human T cells isolated directly from peripheral blood (Martin et al., 1997) or after anti CD3 activation (Liszewski et al., 2013) (also reviewed in (Dunkelberger and Song, 2010;Kemper and Kohl, 2013)). However, activated T cells from patients with a severe inflammatory skin disease (atopic dermatitis) did express C3aR, whereas no expression of C3aR was found in T cells from patients with mild inflammatory skin diseases or from healthy subjects. When C3a was added to C3aR expressing CD4+ and CD8+ T cells from the patients with severe inflammatory skin disease, a transient calcium flux within the T cells was observed,

whereas this did not occur in C3aR-negative T cells (Werfel et al., 2000), validating the presence of a functional C3aR and suggesting a role for C3a on T cell function.

Thus, C3a certainly has a proinflammatory role on T cell function, perhaps as could be expected from activation of the powerful complement protective effector system during infection. Indeed, C3aR, but not C5aR, was recently found to be required for specific CD4+ and CD8+ T cell responses in lung-draining lymph nodes and critical for mouse survival during *Chlamydia psittaci* infections. It remains to be determined whether C3a was acting on the T cells directly or indirectly via APC (Bode et al., 2012;Dutow et al., 2014). Interestingly, another study investigating the role of C3 (but not specific C3 fragments) found that during primary *Listeria monocytogenes* infection, a lack of C3 dramatically reduces the proliferation of both CD4+ and CD8+ T cells. Furthermore, anti CD3-induced proliferation of CD8+ T cells isolated from spleens of *Listeria monocytogenes*-infected $C3^{-/-}$ mice was significantly lower than CD8+ T cells from C3 sufficient mice, suggesting that reduced CD8 T cell responses to *Listeria monocytogenes* in $C3^{-/-}$ mice may be due at least in part to the absence of direct C3 effects on CD8+ T cells (Nakayama et al., 2009). The effect of exogenously added C3a or C3 on the induced proliferation of the cells from $C3^{-/-}$ mice would determine if this were a direct effect of these ligands or of downstream activation products. Going forward, experiments using mice with inducible and conditional deletion of C3aR (and C5aR) selectively in T lymphocytes will verify the function of these receptors in T cells (at least in mice) in disease models. Complementary studies with adoptive transfer of WT T cells into $C3aR^{-/-}$, $C5aR^{-/-}$ and $C3aR^{-/-}$ $C5aR^{-/-}$ mice should help resolve some of the remaining details which could be important for the therapeutic application of these findings for either enhancing the immune response to infection or suppressing autoimmune inflammation.

In addition to the effect of C3a on the induction of responses upon activation of T cells (whether by anti CD3 and anti CD28 stimulation or using the antigen specific OT-I/II transgenic mouse system), some of the above mentioned studies also provided evidence of a "tonic" role for locally (T cell) synthesized C5a and C3a. A new study has provided quite compelling data that indeed intracellular C3a may have a critical survival function in human T cells, and has provided clues to disparate results seen between mouse and human studies. In a series of intriguing confocal imaging and flow cytometry experiments conducted entirely in human cells, Liszewski and colleagues demonstrated that C3 cleavage to C3a and C3b can occur intracellularly in activated T cells via the protease cathepsin L (CSTL). They showed that within 12 hours of exposure to a (nontoxic) CSTL inhibitor (which prevented intra- and extracellular C3a generation) CD4+ T cells underwent apoptosis associated with reduced mTOR phosphorylation. Cell viability could not be restored by addition of purified exogenous C3a, supporting a role for intracellularly produced C3a in CD4+ T cell survival. Additionally, reduction of intracellular T cell C3aR expression (by siRNA) induced a decrease in mTOR activity and cell viability similar to that induced by the CSTL inhibitor, implying that intracellular C3a generation and C3aR ligation contribute to mTOR activity and overall T cell survival. The presence of a CTSL inhibitor, which eliminated only extracellular C3 cleavage, partially suppressed Th1 and Th17 cytokine responses, which could be partially rescued by adding exogenous C3a to the cell culture media and activating anti CD46, suggesting that cell surface generation of C3a and C3b (a ligand for CD46) contribute to the function of these T cell lineages (Liszewski et al., 2013). Furthermore, T cells from patients with autoimmune arthritis demonstrated elevated intracellular C3a and phosphorylated mTOR with a trend for increased T cell IFN-γ and TNF-α production (Liszewski et al., 2013). Based on surface translocation of C3aR and CSTL induced on T cell

activation (anti CD3) and other supportive experiments using human cells, the authors concluded that *in vivo* TCR engagement would cause the intracellular C3aR and CSTL to be shuttled to the cell surface. Cell surface CSTL would generate extracellular cell surface C3a and C3b, which in turn would engage C3aR and CD46 respectively, leading to induction of T effector functions (Liszewski et al., 2013). Thus, in addition to the intracellular role of C3a on T cell survival, extracellularly generated local C3 cleavage dictates certain T cell lineage responses. Importantly, this model is consistent with the observations that C3-deficient patients lack Th1 cell responses. In addition, this distinction between intracellular and extracellular C3 (especially C3a), explains why some C3-deficient patients (that produce intracellular C3a, but not full length C3) have T cells that proliferate but are deficient in proliferation and survival of IFN-γ^+ T cells (Ghannam et al., 2008;Ghannam et al., 2014).

It is important to distinguish direct effects of C3 on T cells from the indirect, APC-mediated effect of C3a shown to result in enhanced Th17 cells. C3a signaling on murine bone marrow derived dendritic cells has been shown to enhance production of IL-23 and IL-6 and suppress IL-10 production, promoting Th17 differentiation in experimental allergic asthma (Lajoie et al., 2010). The high C3a plasma concentration in humans during acute asthma (Nakano et al., 2003) is consistent with this effect. A study showing that $C3^{-/-}$ mice demonstrated significantly lower graft-versus-host-disease (GVHD) mortality and morbidity after receiving WT bone marrow transplants which was associated with lower numbers of Th17, Th1 and CD4+IFN-γ+IL-17+ T cell subsets in secondary lymphoid organs is consistent with a requirement for host production of C3 to facilitate these T effector populations and subsequent GVHD. Additionally, co-culture of donor CD4+ T cells with recipient $C3^{-/-}$ APCs resulted in significantly reduced Th1 and Th17 responses relative to WT APCs, suggesting that C3

fragments from the APC contribute to APC-mediated T cell subset induction (Ma et al., 2012a). However, these data could also be explained by a lack of local generation of C5a downstream of C3 activation (Kwan et al., 2012) (or both) contributing to T cell effector function and GVHD pathology (See below under C5). Nevertheless, considering all the data, in both humans and mice, C3a has been shown to act both directly on the T cell and indirectly via APC C3aR, triggering T cell activation to fight infection and promote inflammatory diseases.

Although less studied, there are several reports on how C3b affects T cell proliferation. C3b (along with C3a) has been shown to be necessary for proper human Th1 cell function via interactions with CD46 on the T cell (below and reviewed in (Dunkelberger and Song, 2010;Kemper and Kohl, 2013)). However, in addition to direct T cell ligation, C3b bound to tetanus toxin results in enhanced antigen presentation to T cells in mice or human transformed cell lines (Arvieux et al., 1988;Serra et al., 1997). One study demonstrated that C3 is deposited on the surface of murine peritoneal macrophages in response to γ-inulin injection, which then enhanced macrophage dependent antigen specific T cell proliferation 2.5-fold when compared with macrophages of untreated animals. This effect was diminished by the presence of antibodies against C3 fragments, suggesting that C3 is enhancing macrophage-mediated antigen dependent T cell proliferation (Kerekes et al., 2001). In a more recent study, Botto and colleagues found that a complete lack of C3 significantly decreased overall T cell expansion induced by dendritic cells that had ingested apoptotic cells *in vivo*, but did not selectively affect the proliferation of particular T cell lineages relative to WT animals (Baudino et al., 2014). Additional experiments in Factor I deficient animals suggested that the likely fragment involved was iC3b or C3dg (not C3b). However, CR3, the common receptor for iC3b, was not required, thus suggesting an alternative receptor or mechanism is involved. The presence of the C3

fragments delayed colocalization of the apoptotic cells with lysosomes (i.e., delayed phagosome maturation) (Baudino et al., 2014), suggesting that this C3 associated delay facilitates MHC class II loading and enhanced antigen presentation (Delamarre et al., 2006;Baudino et al., 2014). Interestingly, in humans, iC3b opsonization of apoptotic cells ingested by monocyte derived macrophages results in macrophage polarization towards an anti-inflammatory phenotype, with NFkB inhibition and increased IL-10 secretion (Amarilyo et al., 2010). This suggests that, in contrast to the effect of an iC3b on apoptotic cells in mice enhancing antigen presentation by dendritic cells (Baudino et al., 2014), human APC expression of iC3b receptors could be responsible for iC3b- triggered anti-inflammatory phenotype in human macrophages. Taken together, these data demonstrate that complement fragments C3a and C3b/iC3b have either a direct role or indirect role, via APCs, in promoting T cell activation during infection, inflammatory disease and homeostasis, although additional studies confirming and extending these data in human systems are still needed.

Complement fragment C5a

Hereditary complement component C5 deficiency was first described in humans nearly 40 years ago. Serum from these individuals has a severely restricted ability to induce chemotaxis and an inability to generate the C5b-9 membrane attack complex. Consequently, these patients typically suffer from severe recurrent infections, especially *Neisseria* species (Rosenfeld et al., 1976). C5a, along with C5b, is a product of C5 proteolytic cleavage by C5 convertase and is a very inflammatory molecule with potent chemotactic and anaphylatoxic properties (Klos et al., 2009). However, whether C5a (via the C5a receptor) acts directly on T cells or via APCs acting

on T cells has not been fully established, and there remain some conflicting reports on whether C5aR is expressed on the surface of T cells (particularly human T cells).

In mice, both C5aR and C3aR mRNA were found to be upregulated on both T cells and DCs during T cell – APC cognate interactions in response to OVA peptide in OT-II cells (Strainic et al., 2008). Moreover, IFN-γ gene upregulation induced by anti-CD3 and anti-CD28 stimulation of WT T cells was partially reduced in $C5aR^{-/-}$ T cells. In addition, as discussed above, the same group demonstrated that an absence of both C3aR and C5aR signaling in CD4+ T cells resulted in enhanced production of IL-10 and enhanced TGF--β1 expression which then mediated induction of Foxp3+ regulatory T cells (iTreg) with robust suppressive activity in mice(Strainic et al., 2013). A separate report demonstrated that signaling through both C3aR and C5aR on mouse thymus-derived CD4+Foxp3+ natural Treg cells (nTreg) resulted in lowered nTreg cell Foxp3 expression and abrogated nTreg cell function (Kwan et al., 2013). Chemical antagonism as well an antibody blockade of both C3aR and C5aR in combination with TGF--β1 was shown to induce functional human iTreg cells from naïve T cells incubated with autologous DCs, anti-CD3 and IL-2 (this was not observed with TGF--β1 alone). That is, treating human CD45RA+CD25CD4+T cells with C3aR and C5aR antagonists or monoclonal anti-C3 and -C5, Strainic and colleagues demonstrated the acquisition of suppressive activity (Strainic et al., 2013) (and see Figure in (LeFriec G. et al., 2013)). In contrast, however, in mice receiving a *Plasmodium yoelii* 17XL (malaria) specific whole-killed blood-stage vaccine, C5aR signaling was found to be critical for the malaria specific CD4+ T cell response, not through direct effects on the expansion of the regulatory (CD4+CD25+Foxp3+) T cells, but rather due to a deficiency in the ability of $C5aR^{-/-}$dendritic cells to stimulate CD4+ T cells (Liu et al., 2013).

However, in one recent conflicting report looking at C5aR expression in murine T cells, Song and colleagues engineered a GFP knock-in mouse in which GFP was inserted such that both GFP and C5aR were expressed under the C5aR promoter. They demonstrated that GFP is highly expressed in blood, spleen, and bone marrow Gr1+CD11b+ cells and moderately expressed in circulating CD11b+F4/80+ leukocytes and peritoneal macrophages as expected; however no GFP was detected on resting T cells, T cells activated via TLR or antigen (OVA peptide in OT-I and OT-II transgenics), splenic myeloid or plasmacytoid dendritic cells (Dunkelberger et al., 2012). Thus, future experiments using mice lacking C5aR in T lymphocytes only (conditional knockout) or adoptive transfer of WT T cells into $C5aR^{-/-}$ mice should further clarify the role of these receptors expressed in T cells.

In several studies, C5aR signaling in APCs has been shown to modulate T cell function indirectly. A recent report evaluated the role of C5aR (plus possibly C3aR) signaling in specific cell types during autoimmune arthritis. Elevated C3a and C5a levels (triggered by complement activation initiated by injected mannan or beta-glucan particles into mice) was shown to promote production of proinflammatory cytokines by macrophages, resulting in spontaneous differentiation of CD4+ T cells to Th17 cells, consequently initiating the development of autoimmune arthritis in autoimmune prone SKG mice (Hashimoto et al., 2010). Naïve murine CD4+ T cells co-cultured with autologous peritoneal macrophages in the presence of C5a and TGF-β differentiated into Th17 cells but not Th1 cells. In addition, *in vivo* macrophage depletion inhibited autoimmune arthritis in SKG mice suggesting that C5aR signaling on macrophages is necessary (but not necessarily sufficient, as the authors did not rule out the contribution of C3aR) for the effect on Th17 differentiation and subsequent development of arthritis in this model (Hashimoto et al., 2010). Schmudde and colleagues demonstrated that ovalbumin antigen uptake

and processing as well as IL1β, IL-6 and IL-23 production was impaired in $C5aR^{-/-}$ murine bone marrow derived dendritic cells, such that when these cells were adoptively transferred to WT mice, reduced Th17 cell differentiation associated with accelerated activated T cell death resulted (Schmudde et al., 2013). Together these studies provide substantial evidence for a role for C5a in modulating T cell function indirectly by altering APC functional state.

In contrast, far fewer studies have evaluated a direct role of C5aR on human T cell function. Aside from the studies referenced above in which both C5aR and C3aR were antagonized in human T cells (Strainic et al., 2013;van der Touw et al., 2013), an older study demonstrated that C5aR mRNA and protein expression were detected in human T cells after PHA stimulation (Nataf et al., 1999), but follow-up studies corroborating this result are lacking. Further work needs to be done to evaluate whether C5a interacts directly with T cells to significantly modulate induction and stability of iTreg in humans and to specifically verify human signaling pathways for future therapeutic manipulation.

Complement regulator MPC/CD46

CD46 (previously known as membrane co-factor protein, MCP) serves as a cofactor for Factor I, the enzyme which inactivates host cell surface C3b and C4b (and thus the C3 convertases) to prevent detrimental ongoing complement activation and lysis of host cells (Liszewski et al., 1991). In addition, there are a number of studies demonstrating a clear role for CD46 in regulating T cell mediated immune responses *in vivo* (Karp et al., 1996) and T cell activation *in vitro* ((Kemper et al., 2003) and reviewed in (Yamamoto et al., 2013;Kemper and Kohl, 2013)) in human systems. Surprisingly, Jagged 1, a Notch family member, is a physiologic ligand for CD46 and appears to be sequestered from Notch 1 (by this interaction with CD46)

until C3b is locally generated, binds to CD46, releasing the constraint on Jagged 1 signaling and thereby contributes substantially to the induction of Th1 cells in humans (LeFriec G. et al., 2012). Furthermore, approximately 30% of individuals deficient in CD46 develop common variable immunodeficiency (CVID) (Fremeaux-Bacchi et al., 2006). Thus, CD46 appears to have regulatory as well as inflammatory functions (at least in human cells as discussed above).

Interestingly, a recent study identified a potential mechanism for this dual function of CD46 on T cells. While original reports described CD46 as inducing a Tr1-like, IL-10 producing phenotype (Barchet et al., 2006), continued investigation using primary human cells led to the discovery that a CD46-mediated T cell lineage switch from IFN--γ producing Th1 effector cells to IFN-γ^{+}IL-10^{+} and then IFN-γ^{-} IL-10^{+} Th1 cells in response to T cell antigen receptor (TCR)-mediated activation occurs at higher concentrations of IL-2. As a disease correlate of these *in vitro* studies, they also demonstrated that CD4+ T cells from patients with rheumatoid arthritis are defective in the IFN-γ–IL-10 switch induced by CD46, presumably contributing to a hyper immune state *in vivo* (Cardone et al., 2010). These findings were extended in a study of nTreg cells (that suppress autoactive immune responses) from 152 lupus patients (LeBuanec H. et al., 2011). Specifically, when purified nTregs from active and inactive lupus patients were stimulated with anti CD3 under steady state, they were able to suppress conventional T cells in a cell contact-dependent fashion to the same extent as a healthy donor. This cell contact-dependent suppressive function (i.e., nTreg activity) was, however, diminished by anti CD3/CD28/CD46 stimulation in the presence of IFN-α, conditions which mimicked the elevated C3b and IFN-α associated with *in vivo* SLE inflammatory conditions. Thus while IFN-α plus ligation of CD46 increases IL-10 production by converting both nTreg and conventional T cell (Tconv) to Tr1 (IL-10 secreting) cells to suppress inflammation and prevent tissue damage, during SLE when IFN-α

and TcR activation is excessive, nTreg activity is reduced leaving the individual more susceptible to induction of further autoimmune responses (LeBuanec H. et al., 2011;Kemper et al., 2003).

A couple of recent studies evaluated if this CD46-mediated costimulatory function could be retained in human T cells deficient in C3, if anti CD46 was used in place of the natural ligand (which at the time was unknown but postulated to be C3b). A unique study used purified CD4+ T cells from an individual deficient in complement component C3 treated with anti CD3, anti CD46 and IL-2, resulting in much lower IL-10 secretion relative to C3 sufficient control T cells, i.e., patients deficient in C3 were also deficient in Tr1 cells (Ghannam et al., 2008). Since the patient's T cells exhibited normal CD46 expression levels, the authors hypothesized that the lack of Tr1 induction (i.e., high IL-10 production) could have resulted from other impaired signaling in C3 deficiency (Ghannam et al., 2008). In a follow-up study using two C3 deficient patients, they found that Th1, but not Th2 responses were impaired in both C3 deficient individuals (Ghannam et al., 2014). However, when the authors reconstituted the C3-deficient T cell cultures with C3a and IL-2, Th1 and Tr1 induction (by anti CD3 and anti CD46) was restored in one of the C3-deficient patient's T cells, but not the other. Thus while the difference between the responses of these two patients remain to be definitively identified, these data, combined with the defective Th1 induction in CD46 deficient individuals suggest that engagement of both C3aR and CD46 are required for the CD46-mediated regulation of human Th1 cells and subsequent Tr1 induction (Ghannam et al., 2014). This molecular pathway is thus substantially different from the requirements for murine Th1 induction, since mice do not express CD46 on most cells and, as described above, C3aR and C5aR (with C3a and C5a) contribute to the induction of IFN-γ producing T cells in mice.

Complement regulator DAF/CD55

Decay accelerating factor (DAF, CD55) binds C3b and C4b to accelerate the decay of both the alternative and classical pathway convertases, providing protection against amplified C3b deposition and complement activation on the plasma membranes of normal host cells. Genetic defects that result in reduction or loss of erythrocyte DAF lead to paroxysmal nocturnal hemoglobinuria (PNH) (Nicholson-Weller et al., 1983). Additionally, DAF germline mutation results in global DAF deficiency associated with the "Inab phenotype," and Crohn's disease-like enteropathy in two cases (Telen and Green, 1989), but the causal relationship between DAF deficiency and this enteropathy is not clear.

DAF expression was first detected on human T cells nearly 30 years ago (Kinoshita et al., 1985) and later was found to be expressed on both activated and naïve T cells (Christmas et al., 2006). Over the past few decades, DAF, acting either directly on the T cell or indirectly via APCs, has been shown to play either an activating role or suppressive role, respectively, in T cell function. In humans, it was observed that T and B cell DAF and CD59 expression was significantly diminished in SLE patients with lymphopenia (associated with higher titers of autoantibodies) when compared with healthy donors. However the opposite was found in T and B cells from non-lymphopenic SLE patients, suggesting that combined DAF and CD59 deficiency may increase the susceptibility of cells to complement mediated lysis (Garcia-Valladares et al., 2006), but not necessarily pinpointing a role for T cell DAF-induced signaling in modulating T cell survival. However, *in vitro* studies with human T cells provide evidence that DAF does have a costimulatory function leading to T cell proliferation. For instance, one study used primary human T cells to demonstrate that when naïve (CD45RA+) CD4+ T cells were stimulated with either anti CD55/DAF or plate-bound CD97 and anti CD3 in the presence

of IL-2, overall T cell proliferation was enhanced, T cell IL-10 production was substantially enhanced (10-fold) and IFN-γ production was slightly increased (2.5-fold) (Capasso et al., 2006), demonstrating a direct effect of DAF on human T cell function. A subsequent report in which peptide loaded monocytes were cocultured with human T cell clones to approach more physiologic conditions of antigen presentation found that blocking the interaction between DAF on human monocytes and CD97 on human T cells results in inhibition of T cell proliferation and IFN-γ production (though IL-10 production was not measured in this study), presumably via blocking a contribution to stabilization of the immunological synapse. While the mechanism for both of these findings remains to be defined, the data suggest that CD97 and DAF are another co-stimulatory pair involved in promoting T cell activation (Abbott et al., 2007). That is, the functional consequences of CD55 do not seem to be correlated with its decay accelerating activity of cell bound C3b and thus the supply of complement activation fragments.

On the other hand, murine DAF seems to exert its effects via regulation of C3 convertase activity (i.e., C3a and/or downstream C5a). For example, DAF has been shown to play a role in preventing allograft rejection *in vivo*. One study demonstrated that in mice, donor $DAF^{-/-}$ allograft hearts were rejected faster relative to WT allografts, and this rapid rejection in the absence of DAF was dependent on allograft production of C3, and subsequent C3-dependent proliferative / pro-survival effects on a higher number of IFN-γ producing, transplant specific T cells. Interestingly, bone marrow derived cells in the $DAF^{-/-}$ donor allograft hearts were found to drive the kinetics of graft rejection, consistent with the effect being related to deficient regulation of C3 cleavage by the local $DAF^{-/-}$ APC and thus enhanced T cell function in the transplant (Pavlov et al., 2008). This observation was supported by similar lack of rejection with C3-deficient APCs. However, the decrease in T cell IFN-γ production observed in the DAF-

sufficient murine system containing heart-BM chimera cells (relative to $DAF^{-/-}$) could be a consequence of DAF modulating T cell function indirectly via APCs as opposed to the direct DAF-CD97 costimulatory function observed in human T cells.

In another example, mice deficient in DAF exhibited elevated T cell IFN-γ and IL-2 as well as reduced IL-10 and enhanced T cell responses when these T cells were pooled and re-stimulated with antigen *in vitro*, consistent with a lack of control of C3 convertase activity, which would lead to increased local C3a and C3b generation and thus Th1 activation. *In vivo* studies were consistent with this interpretation, as T cells from $DAF^{-/-}$ mice produced elevated levels of IFN-γ and IL-2 relative to those of WT mice and $C3^{-/-}DAF^{-/-}$ mice produced levels of IFN-γ and IL-2 that were similar to those of T cells from WT mice (Fang et al., 2011). This enhanced T cell cytokine production in the absence of DAF was dependent on APC expression of C3aR and C5aR and was correlated with decreased surface PD-L1 and increased CD40 expression, suggesting an indirect role of DAF in suppressing T cell activation due to limiting the generation C3 cleavage products such as C3a and subsequent diminished C5 convertase activity (e.g., C3 and C5 fragments alter APC function to promote inflammatory T cell activity) (Fang et al., 2011). Additionally, in a T cell–dependent experimental autoimmune encephalomyelitis (EAE) model (Liu et al., 2005), a deficiency of DAF accelerated disease. When the complement system was disabled by a genetic deficiency of C3 in the $DAF^{-/-}$ mice, however, the T cell lineage skewing and exacerbation of T cell-dependent EAE was diminished, demonstrating that again in this study, DAF is regulating T cell-dependent EAE via its regulation of the complement pathway. Similarly, during LCMV infection, mice deficient in DAF had substantially greater LCMV antigen specific T cell expansion and these T cells exhibited greater killing capacity and cleared the virus more efficiently. However when either C3 or C5aR (CD88)

was also absent, the $DAF^{-/-}$ mice no longer exhibited enhancement of CD8+ T cell immunity, once again suggesting that DAF is regulating T cell immunity in a complement pathway dependent fashion (Fang et al., 2007).

In yet another murine model, DAF has also been shown to suppress interphotoreceptor retinoid-binding protein (IRBP) specific Th1 and Th17 responses and disease severity in murine experimental autoimmune uveitis (EAU) in a complement pathway-dependent manner. Both EAU incidence and histopathology scores were significantly greater in $DAF^{-/-}$ mice with the EAU disease, and these mice exhibited up to 7-fold greater Th1 and 4-fold greater Th17 responses against IRBP. Importantly, mice treated with soluble DAF protein exhibited decreased IRBP specific Th1/Th17 responses and were protected from retinal injury relative to controls. This DAF-mediated regulation of enhanced Th1 and Th17 responses and subsequent EAU disease was found to be due to DAF expression on both APCs and T cells, which then modulated C3, factor B, factor D, and C5 synthesis during their cognate interactions, consistent with an earlier report from Strainic and colleagues (Strainic et al., 2008) and implying that DAF is preventing IRBP specific Th1/Th17 responses in complement pathway-dependent manner in this study.

Experiments as described above performed with mice deficient in DAF have identified a role for the protein in indirectly regulating T cell activation via either DAF on APCs and/or T cells via regulation of complement activity. However, it remains to be seen whether DAF can function similarly on human cells. Furthermore, studies using human APCs and T cells have uncovered a direct costimulatory role for DAF on T cells (via CD97 ligation), but a direct function for DAF on murine T cells has not been identified. Thus, future studies should aim to explore whether DAF plays a C3-dependent role on human T cell function and determine

whether the observed human costimulatory mechanism of DAF on T cells is also occurring in mice as well as humans for the purposes of laying the groundwork for mechanistic studies in murine models testing of therapeutic strategies.

Summary: C3b, iC3b, C3a, C5a, CD46 and DAF modulation of T cell function

Complement activation products C3a and C5a have been extensively studied in mice and have a clear role in directly and indirectly promoting T cell activation and proliferation, and as such promote/exacerbate allograft rejection, autoimmunity, and fighting infection. The C3a-C3aR axis plays similar roles in T cell survival and activation of T cells in humans, and thus suggests possible therapeutic interventions in either enhancing or suppressing Teff activity. In the case of C5a, less corroborative data have been collected using human cells making it difficult to definitively translate the murine results to human T cell immunity and disease. *In vitro* experiments with anti C5 antibody, Eculizumab, may provide some insight (Wong et al., 2013), but studies in C5-deficient patients would also be valuable in addressing this issue. However, studies with human T cells from both healthy donors and patients afflicted with CVID or lupus have identified a clear role for CD46 (not expressed on most cells in mice) as an inflammation sensor on human T cells which can depend strongly on the cytokine milieu. A novel surface interaction with the Notch 1 family Jagged 1 that appears to participate in the control of CD46-mediated modulation of T cell function is another potential area for therapeutic modulation targets.

The effect of the complement regulator DAF has been studied on human and murine T cells, highlighting a direct role for the protein in co-stimulation of human T cell proliferation via CD97 while identifying an indirect role in regulating murine T cells (either acting on APCs

and/or by regulating generation of C3a and C5a). More studies are needed to understand whether DAF can influence human cells in an indirect fashion (e.g., in a C3-dependent role vs. CD97 ligation) as well. *In vitro* co-culture experiments of APCs and T cells with and without purified C3, C3 fragments and other complement components and/or DAF inhibitors may help elucidate whether such pathways exist.

In summary, although progress has been made towards understanding the role of complement proteins on T cell function during homeostasis and disease, more data are required to pinpoint the mechanisms involved and therefore identify targets for therapeutic intervention in human diseases. It is important to note that for several of the proteins—C3a, C5a and DAF, and CD46 (which is not even expressed in most mouse cells)—there are substantial differences between the data in human and mouse systems (in some cases with opposite effects) (see Figure 2 in (Heeger and Kemper, 2012)). This underscores the importance of carrying out experiments in human cells or verifying that mechanistic pathways derived from murine in vivo systems are applicable to human systems, to facilitate successful translational applications to the humans such as therapeutic development (Seok et al., 2013). Thus, I have focused my studies on the role of complement C1q on T cell function in human systems, as the data gathered from such experiments will be more readily translated for therapeutic and/or preventative applications in human disease.

C1q modulation of T cell function

In addition to initiating the classical complement cascade (i.e., when C1r and C1s are present to initiate the formation of C3-convertase, C4b2b), the C1q protein is expressed and functions in the absence of C1r and C1s in tissue (Benoit et al., 2013;Bensa et al., 1983). In this

context, C1q can bind apoptotic cells to enhance ingestion by phagocytes before membrane integrity of the dying cell is lost. It has been known for some time that C1q-deficient individuals present with systemic lupus erythematous (SLE) with extremely high (93%) penetrance (Botto and Walport, 2002) and that $C1qa^{-/-}$ mice can develop autoantibodies and an SLE-like disease (Botto et al., 1998). Furthermore, C1q knockout mice exhibit accelerated rejection of minor histocompatibility disparate skin grafts and resistance to induction of tolerance (Baruah et al., 2010).

While one may expect that a lack of C1 would lead to less C3 fragment deposition and thus less effective antigen presentation resulting in a diminished immune response (see above and (Baudino et al., 2014)), the fact that the lack of C1q inevitably leads to SLE rather than suppressing autoimmunity supports a direct role for C1q in APC polarization seen in the human studies (Fraser et al., 2009;Benoit et al., 2012). C1q is known to be expressed in the absence of C1r and C1s in tissue (Benoit et al., 2013;Bensa et al., 1983), is synthesized by macrophages, and is induced in response to local injury (Dietzschold et al., 1995;Goldsmith et al., 1997) as reviewed in (Alexander et al., 2008). As a result, C1q predominance in perturbed tissue may serve to accelerate the ingestion of dying cells and the resolution of inflammation (as reviewed in (Elkon and Santer, 2012)), and therefore in this context C1q may be considered an immunological “rheostat” or homeostatic regulator. Thus, the evaluation of C1q specifically (in a serum-free system) represents an important approach for evaluating the effects of C1q interaction with macrophages in the tissue during low levels of inflammation such as would occur during mild sterile injury or with decreasing danger signals to promote contraction of immune cell populations.

The mechanism for how C1q may avoid the initiation SLE in normal individuals (or propagation of the disease in SLE patients) is only beginning to be elucidated. Korb and Ahearn demonstrated that C1q binds apoptotic cells through its globular head domain (Korb and Ahearn, 1997), which leaves the collagen like domain available for interactions with phagocytic cells (Bobak et al., 1987). Our group and several others have studied how the C1q interaction with antigen presenting cells (APCs) affects the phenotypic and functional state of the APC, polarizing it towards an anti-inflammatory function with elevated levels of IL-10 and decreased pro-inflammatory cytokines, (reviewed in (Elkon and Santer, 2012;Fraser et al., 2009)) which, by modifying the microenvironment could potentially be modulating T cell activation. Recently, Lu and colleagues found that primary human dendritic cells interacting with immobilized C1q exhibited an anti-inflammatory functional phenotype and suppressed allogeneic CD4 Th1 and Th17 lineages (Teh et al., 2011), both of which have been found to be elevated in SLE patients (Liu and Davidson, 2012).

The capacity of C1q to modulate T cell function has also been studied in murine models although there appear to be some differences from the primary human systems described above. For example, Cutler et al demonstrated that in $C1q^{-/-}$ mice, antigen-specific T cells exhibited a significant reduction in IFN-γ production compared to control mice (Cutler et al., 1998). Similarly, a more recent study from the same group used DCs from $C1q^{-/-}$ mice and found that in this context, exogenously added C1q augments the production of IL-12 by DCs and increases the number of CD4+IFN-γ+ (Th1) and CD8+IFN-γ + T cells in response to DC CD40 ligation (Baruah et al., 2009). While these experiments suggest differences between mouse and human systems, they may also, or alternatively, reflect a difference between C1q modulation of T cell function in mice with lifetime genetic deficiency of C1q, and thus all the other consequences of a

lack of that molecule, such as the recently described deficiency of synaptic pruning and plasticity during brain development (Bialas and Stevens, 2013). Furthermore, the results in the $C1q^{-/-}$ model alone cannot distinguish between the function of the C1 complex (C1q + C1r + C1s) and thus a deficiency of the complement fragments resulting from the activation of the classical complement cascade versus a deficiency of C1q alone.

The presence of C1q receptors on T cells themselves and the potential for specific consequences of an interaction of C1q with T cells has not been fully defined. One study from two decades ago demonstrated (by western blot) that human T cell expression of a 60 k Mr C1q receptor (cC1qR) which interacts with the collagen-like domain of the molecule (Malhotra et al., 1990). The expression of this C1qR is up-regulated by mitogens that induce T cell proliferation, although the addition of C1q prior to the addition of mitogen induced an anti-proliferative signal (Chen et al., 1994) suggesting that the receptor must already have been present on the cell. This report presents an intriguing possibility for a direct effect of C1q on T cell function, as C1q was added to T cells directly in the media. However, as such this soluble "monomeric C1q" was not necessarily interacting with the T cells in a multivalent fashion as is usually seen to be critical for stable C1q-cell interactions. In addition, the assays were all conducted in cultures containing 10% fetal calf serum (FCS), so the interaction of exogenously added C1q with other serum proteins present cannot be ruled out as playing a contributing role in the C1q effect observed. Although the data thus far suggest that C1q probably modulates T cell activation, more work is needed to elucidate whether and how C1q directly interacts with T cells to modulate their function, whereas how some of the *in vivo* mouse T cell (Cutler et al., 1998) studies to date fit into the human and mouse C1q deficient phenotype scenario is not completely clear at this time (Botto et al., 1998;Botto and Walport, 2002).

In this thesis, I have developed a novel experimental system consisting entirely of primary human cells in which to investigate whether and how C1q may modulate APC-mediated T cell function. I propose that studies evaluating the interaction of C1q on a physiologically relevant target with primary human APCs followed by co-culture with T cells approximate the sort of environment that may be present during low levels of inflammation in the tissue, and would highlight the immunoregulatory role C1q may be playing in this context (reviewed in (Elkon and Santer, 2012)). Analysis of these interactions and consequences could contribute to the understanding of why humans deficient in C1q develop lupus nearly 100% of the time.

Objective of the Present Study

In the experiments reported in this thesis, I investigate the effect of C1q bound to autologous apoptotic cells on primary human Mϕ and DC functional phenotype and subsequent Mϕ and DC–mediated human CD4+ and CD8+ T cell subset proliferation. This novel experimental system recapitulates a physiological context relevant to the initiation and propagation phases of SLE. Moreover, this approach is unique among studies addressing the issue of macrophage direction of T cell responses and avoids the caveats in previously published studies performed in mice, with transformed/foreign cell lines and with non-physiologic presentation of the C1q molecule. Pinpointing the molecules involved in the C1q-mediated modulation of antigen-presenting cell (APC) and consequent T cell responses could identify critical C1q-mediated autoimmune avoidance pathways spanning the innate and adaptive immune systems and could lead to identification of targets for preventative and therapeutic intervention.

Chapter 2 — Complement Protein C1q Directs Macrophage Polarization and Limits Inflammasome Activity during the Uptake of Apoptotic Cells

Abstract

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 auto-immune diseases characterized by auto-antibodies to self proteins and aberrant innate immune cell activation likely due to impaired clearance of apoptotic cells. Here, a novel autologous system using primary human lymphocytes and monocyte-derived macrophages (Mϕ) was applied 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 Mϕ. Specifically, C1q increased STAT1 phosphorylation and sequentially induced type I interferons (IFN-s), IL-27 and IL-10 in LPS-stimulated Mϕ and IL-27 in Mϕ when incubated with AL conditioned media. Co-incubation with C1q tails prevented the induction of type I IFN-s and IL-27 in a dose dependent manner and neutralization of type I IFN-s partially prevented IL-27 induction by C1q. Finally, C1q decreased procaspase-1 cleavage and caspase-1 dependent cleavage of IL-1β suggesting potent inhibitory effect of C1q on inflammasome activation. These results identify specific molecular pathways induced by C1q to suppress macrophage inflammation providing potential therapeutic targets to control macrophage polarization, and thus inflammation and autoimmunity.

Introduction

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 pro-inflammatory 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 (Bohlson et al., 2007;Sjoberg et al., 2009). Complement functions as an important humoral defense system to sense danger by recognizing pathogen associated molecular patterns (PAMPs) but is also activated by damaged 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 (Alexander et al., 2008;Sjoberg et al., 2009). A causal link between complement deficiency and systemic lupus erythematous (SLE) involves in part the role of complement in physiological waste disposal mechanisms, in particular clearance of dying cells (Manderson et al., 2004). While activation by all three complement pathways can contribute to enhanced uptake of apoptotic cells by phagocytic cells (Nauta et al., 2004;Kemper et al., 2008;Xu et al., 2008;Fraser et al., 2009;Fraser and Tenner, 2010), 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 over 90% of individuals with genetic deficiency of C1q developing severe SLE (Pickering et al., 2000).

C1q is known to play a prominent nonredundant tissue specific role in the clearance of apoptotic cells *in vitro* and *in vivo* (Korb and Ahearn, 1997;Ogden et al., 2001;Vandivier et al., 2002;Taylor et al., 2000;Botto et al., 1998). C1q binds to apoptotic cells and cellular debris through its globular heads(Korb and Ahearn, 1997;Navratil et al., 2001) and to phagocytic receptors through its collagen tails (Klickstein et al., 1997;Bohlson et al., 2007). While at first thought to be primarily of liver origin, C1q is predominantly synthesized *in vivo* by peripheral tissue macrophages and dendritic cells (Petry et al., 1991;Castellano et al., 2010) and by myeloid cells *in vitro* (Fraser et al., 2009;Tenner and Volkin, 1986;Castellano et al., 2004a;Bensa et al., 1983). While C1q is most often bound to C1r and C1s in the circulation (Ziccardi, 1982), 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* (Dietzschold et al., 1995;Goldsmith et al., 1997;Benoit et al., 2013) and reviewed in (Alexander et al., 2008), 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 down-regulation of pro-inflammatory cytokines upon TLR4 stimulation by LPS (Fraser et al., 2006;Yamada et al., 2004).

Earlier work in the Tenner Lab demonstrated 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 (Mϕ) and dendritic cells (DC) (Fraser et al., 2009). 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 (Fraser et al., 2009). However, because several C1q receptors have been identified and none has been shown to specifically mediate C1q-enhancement of phagocytosis of apoptotic cells (Vandivier et al., 2002;Norsworthy et al., 2004;Bohlson et al., 2007;Ramirez-Ortiz et al., 2013), the intracellular signaling pathways engaged upon interaction of C1q with phagocytic cells remain to be fully elucidated. In addition, since 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, a unique system was developed that uses primary human autologous lymphocytes and Mϕ to characterize the effect of C1q on Mϕ gene expression profiles during the uptake of autologous apoptotic cells, a more physiologic system than transformed cell lines as a source of apoptotic cells. The results show that C1q bound to autologous apoptotic lymphocytes (AL) significantly modulates the response of Mϕ to TLR ligation 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.

Material 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 (rh) M-CSF and IL-2 were from PeproTech. ATP was from Sigma-Aldrich. Mouse IgG1 antibodies

were from R&D Systems and anti-human IFN-α and β antibodies were from PBL Biomedical Laboratories. Human serum albumin (HSA) used for elutriation was obtained from Talecris Biotherapeutics. Ultra-pure LPS was from List Biological Laboratories Inc. C1q was isolated from plasma-derived normal human serum by ion-exchange chromatography, followed by size-exclusion chromatography according to Tenner et al. (Tenner et al., 1981) and modified as described (Young, Jr. et al., 1991). C1q tails were prepared as described (Guan et al., 1991). All C1q preparations showed equivalent purity (determined by SDS-PAGE and Coomassie staining) and have less than 0.03 EU/ml endotoxin by Limulus Amoebocyte Lysate clot assay (Lonza).

Cell isolation and culture

All blood samples were collected into CPDA1 at the UCI Institute for Clinical and Translational Science in accordance with guidelines and approval of the University of California Irvine (UCI) Institutional Review Board. Human peripheral blood lymphocytes and monocytes were isolated by counterflow elutriation using a modification of the technique of Lionetti et al. (Lionetti et al., 1980) as described previously (Bobak et al., 1986). 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+ and greater than 90% of the monocyte fraction was CD11b+. Lymphocytes were maintained for 7 days in RPMI1640, 10% FCS, 2 mM L-Glutamine and 1% penicillin/streptomycin (complete media) containing 50 U/ml rhIL-2 and then γ-irradiated (10 Gy) and maintained overnight in complete media or media without FBS to generate EAL (Annexin V+/PI-) or LAL (Annexin V+/PI+), respectively. AL were considered in an early stage (EAL) if PI incorporation was lower than 20% and considered to be in late apoptosis if PI incorporation ranged from 21 – 60%

(Figure 2.1). In some experiments, lymphocytes were pre-labeled with a red membrane cell tracker (PKH26 from Sigma-Aldrich) according to manufacturer's instructions. Mϕ were generated from monocytes by culture for 8 days in complete media containing 25 ng/ml rhM-CSF. For every experiment, apoptosis (apoptosis detection kit from BioVision) and Mϕ phenotype were assessed by flow cytometry.

C1q binding assay

EAL and LAL were incubated with 150 μg/ml purified human C1q for 1 h in PBS/1% HSA at 37˚C. Binding of C1q was assessed for every experiment by flow cytometry using a monoclonal antibody against C1q (Quidel) and FITC-anti mouse IgG (Jackson ImmunoResearch Laboratories). For each experiment, C1q binding was greater than 50% for EAL or LAL (Figure 2.2).

Uptake assay

PKH26-labeled or unlabeled EAL and LAL, pre-coated or not with C1q were incubated with Mϕ at a 5:1 ratio for 1 h in phagocytosis buffer (RPMI1640, 25 mM HEPES and 5 mM $MgCl_2$). For uptake quantification, cells were washed, harvested with Trypsin/EDTA and stained with CD11c-FITC antibodies for flow cytometry analysis or fixed with 3.7% paraformaldehyde and stained with FITC-phalloidin (InVitrogen) according to 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, Mϕ 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 manufacturer's instructions by the UCI Genomics High Throughput Facility at University of California, Irvine. Data processing and analysis were performed using JMP Genomics 5.0 software (SAS Institue Inc.). Briefly, inter-array median correction was used to normalize signal intensities. Significant differences in gene expression compared to unstimulated Mϕ were identified by ANOVA test using Holm multiple testing method and a false positive rate (alpha error) of 0.05. Hierarchical clustering (Pearson correlation coefficient-based heat map using complete linkage method) was performed using JMP genomics and TMeV (Saeed et al., 2006). Functional classification was performed using DAVID software (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009) and pathway network analysis and visualization was performed using Cytoscape (Cline et al., 2007). All data were submitted to the MIAME-compliant (Brazma et al., 2001) database Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, accession number GSE30177).

Reverse transcription and quantitative real-time PCR (qRT-PCR)

The cDNA synthesis was carried out with 100 ng of total RNA and the M-MLV reverse transcriptase RT (Invitrogen) as previously described (Benoit and Tenner, 2011). 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 was determined as follows: FC = 2^{-}

$^{\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{Target} - Ct_{GAPDH})test - (Ct_{Target} - Ct_{GAPDH})$ unstimulated. Ct values were defined as the number of cycles at which the fluorescence signals were detected (Schmittgen and Livak, 2008).

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 manufacturer's instructions.

Detection of cleaved caspase-1

After uptake, Mϕ 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. Mϕ were then incubated with Green FLICA™ Caspase-1 probes (ImmunoChemistry Technologies) and Cell Tracker™ Blue CMF_2HC (InVitrogen) 1 h before the end of the stimulation. Mϕ were then washed, fixed and analyzed immediately by confocal microscopy as described above.

Western blot

For detection of inflammasome components, Mϕ 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, Mϕ were stimulated with LPS for 18 h and ATP was added during the last 3 h of LPS stimulation. Mϕ culture supernatants were concentrated using 10 kDa Amicon columns (Millipore). Mϕ 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

antibodies against NLRP3 (NLR family pyrin domain containing 3, Enzo Life Sciences), caspase-1 (Cell Signaling), ASC (apoptosis-associated speck-like protein, Medical & Biological laboratories), IL-1β (clone 3ZD form the NCI Biological Resources Branch), pSTAT1 (Tyr701) (Cell Signaling Technologies) and β-actin (Sigma-Aldrich) and secondary HRP-conjugated anti-mouse or rabbit IgG antibodies (Jackson ImmunoResearch Laboratories). The blots were developed using enhanced chemiluminescence plus (ECL+, GE Healthcare) and analyzed using the Nikon D700 digital SLR camera and the ImageJ software as described (Khoury et al., 2010).

Flow cytometry for pSTAT1

Mϕ were incubated with AL for 60 minutes, washed and then stimulated with LPS for 30 minutes. Mϕ were then fixed in 1 ml 3.7% PFA and permeabilized with an equal volume of 90% methanol. Mϕ were stained with isotype control or pSTAT1 (Cell signaling Technologies) primary antibody followed by washing and addition ofAlexa488-conjugated secondary antibody (Invotrogen), after which they were washed and read on a BD FACS Calibur. pSTAT1 MFI was then quantified using FlowJo (Tree Star).

Statistical analysis

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

Results

C1q binds to human apoptotic lymphocytes and enhances their ingestion by Mϕ

To determine how C1q modulates human Mϕ responses during the uptake of apoptotic cells, a unique autologous system was developed (lymphocytes and monocytes being isolated from the same donor) where Mϕ were incubated with C1q-coated apoptotic lymphocytes. Lymphocytes were irradiated to induce apoptosis and maintained overnight in complete media or under serum deprivation to generate early (EAL, AV+PI-) and late (LAL, AV+PI+, secondary necrosis stage) apoptotic lymphocytes, respectively (Figure 2.1).

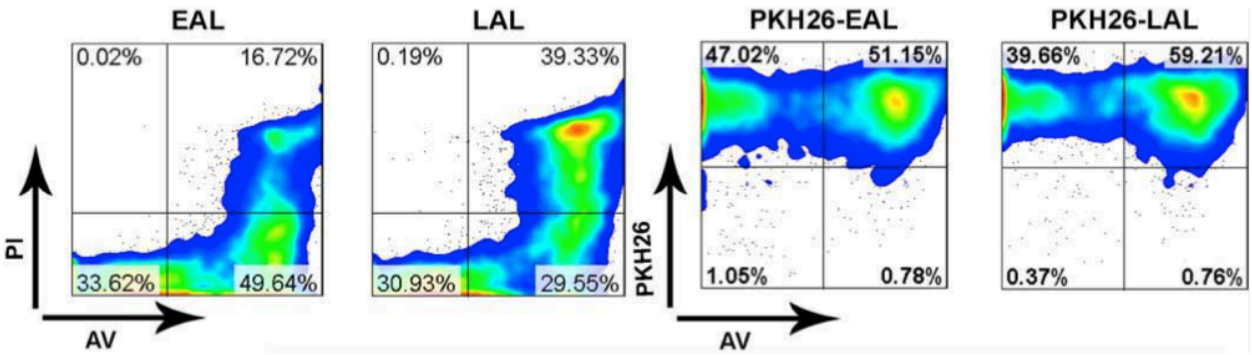
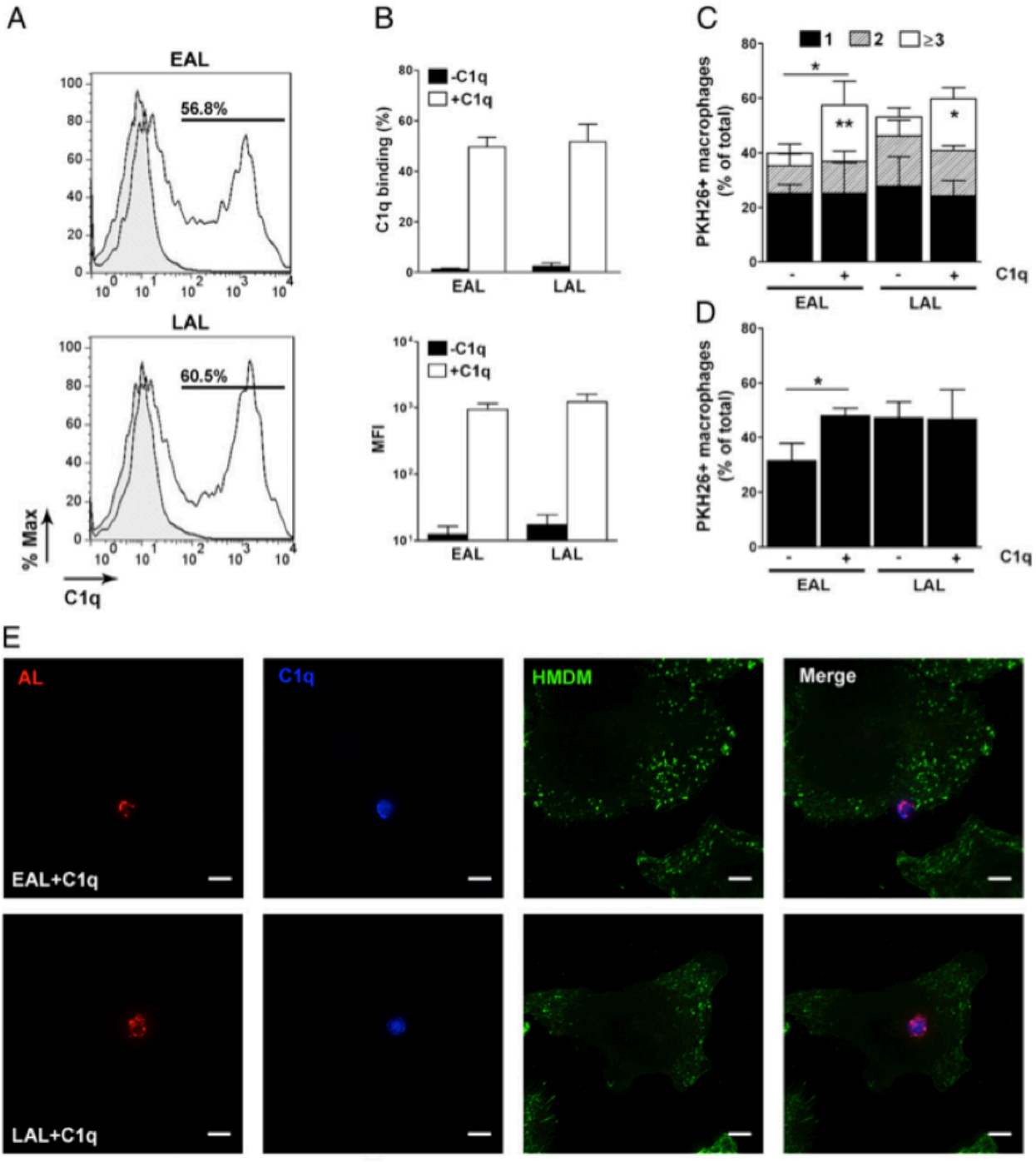


Figure 2.1 – Annexin V and Propidium Iodide Staining of Apoptotic lymphocytes. Lymphocytes, pre-labeled (right) or not (left) with a red cell membrane tracker (PKH26), were incubated for 16 h in media with FBS (EAL, AnnexinV+/PI^{low}) or without FBS (LAL, annexinV+/PI+) after γ- irradiation (10 Gy dose). Apoptosis was determined by flow cytometry using annexinV/propidium iodide staining. AL were considered in an early stage (EAL) if PI incorporation was lower than 20%and considered to be in late apoptosis if PI incorporation ranged from 21 – 60%.

In preliminary experiments, the optimal C1q concentration (150 μg/ml) and incubation time (1 h) to obtain the highest percentage of C1q binding to AL while maintaining C1q concentration at near physiological levels was determined (C1q serum concentration is about 113 ± 40 μg/ml, ranging from 56 to 276 μg/ml depending on the studies and methods (Ziccardi and Cooper, 1978). We found that C1q binds to human EAL and LAL with the same efficiency since about 55 to 60% of both EAL and LAL were C1q positive and have comparable MFI (Figure 2.2). Pre-

labeling of EAL and LAL with the red cell membrane tracker PKH26 did not affect the induction of apoptosis or C1q binding to those cells. The effect of C1q on the uptake of EAL and LAL by Mϕ was then determined by immunocytochemistry and flow cytometry (Figure 2.2).



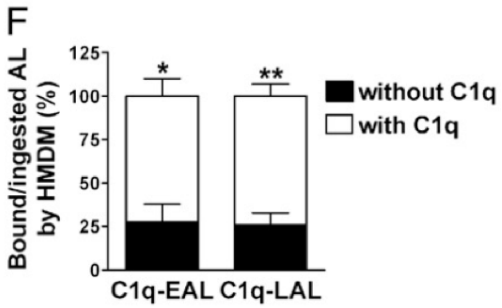


Figure 2.2 – C1q binding to EAL and LAL and effect on the uptake by Mϕ. (A and B) EALs and LALs were incubated without (gray peak) or with 150 mg/ ml C1q (black line) for 1 h, washed, and stained for C1q. Representative FACS plots of multiple experiments are shown. (B) Percentage of C1q binding and anti-C1q mean fluorescence intensity determined by flow cytometry. Results represent means ± SD (n = 5). (C and D) Mϕ were incubated with PKH26-labeled EAL and LAL, preincubated or not with C1q, at a 5:1 ratio for 1 h, washed, and fixed. Cells were stained with FITC–phalloidin and analyzed by confocal microscopy to determine the percentage of phagocytosis and the number of targets per Mϕ (C) or stained with CD11c–FITC Abs and analyzed by flow cytometry (D). Results represent means ± SD (n = 3 different donors), two-way ANOVA. *p< 0.05, **p< 0.01. (E and F) PKH26-prelabeled EAL and LAL (red) were incubated with C1q for 1 h, washed, and then added to Mϕ at a 5:1 ratio for 1 h. Cells were fixed and stained with anti-C1q Abs (blue) and FITC–phalloidin (green) and analyzed by confocal microscopy. Representative micrographs of three independent experiments are shown in (E). Scale bars, 10 mm. (F) Percentage of Mϕ-bound/ingested EAL or LAL bound or not to C1q. Results represent means ± SD (n = 3), two-way ANOVA. *p < 0.05, **p < 0.01.

It is of note that all uptake assays and subsequent stimulation were performed in serum free medium so no other complement proteins than C1q are present in this system in order 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 Mϕ that have ingested at least one EAL but did not increase the percentage of Mϕ that have ingested at least one LAL (Figure 2.2 C-D). However, C1q increased the number of EAL and LAL per Mϕ, particularly the percentage of Mϕ that have ingested 3 or more EAL ($p < 0.01$, Figure 2.2C) or LAL ($p < 0.05$, Figure 2.2C). All together these results showed that C1q binds with the same efficiency to EAL and LAL and enhances their uptake by Mϕ.

To determine if Mϕ preferentially ingest those EAL and LAL bound to C1q, we quantified the number of C1q positive cells after uptake by immunostaining. (Figure 2.2E-F). C1q was detectable on EAL or LAL during the uptake by Mϕ (Figure 2.2E) and more than 70%

of macrophage-bound EAL (72.1 ± 17.6%, $p < 0.05$) or LAL (74.1 ± 12.3%, $p < 0.01$) were C1q positive (Figure 2.2F). 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 Mϕ during the uptake of apoptotic lymphocytes

To delineate the C1q-modulated pathways in macrophages during the uptake of AL, Mϕ were incubated with C1q-bound EAL or LAL 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 HMGB1 or HSP that are normally intracellular but are released when the cells die (Rock and Kono, 2008). Global transcriptional gene expression profiles of Mϕ were analyzed using the Gene 1.0ST array from Affymetrix (Figure 2.3) and validated by qRT-PCR. Hierarchical clustering analysis highlighted groups of genes specifically modulated by the uptake EAL and LAL as compared to LPS alone and also by C1q when bound to EAL or LAL as compared to EAL or LAL in the absence of C1q (Figure 2.3A). Using Gene Ontology (GO) annotation, genes modulated by C1q showed enriched GO biological processes related to signal transduction, G protein-coupled receptor (GPCR) signaling pathway, immune response, homeostasis and macromolecule biosynthesis (Figure 2.3B). 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 (Figure 2.3C-F), some of those genes being

differentially modulated when C1q is bound to EAL or LAL 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 Mϕ only when bound to EAL (Figure 2.3C). When bound to LAL, C1q significantly increased the expression of versican (VCAN), IL-13, a typical M2-driven cytokine, and IL1F7, also known as IL-37, while decreasing the expression of the M1-asssociated chemokine CXCL9 in Mϕ (Figure 2.3C-D). C1q bound to either EAL and 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 IFN-s receptor IFN-AR2 in Mϕ (Figure 2.3C-D). In addition, C1q bound to EAL and LAL modulated the expression of several signaling molecules of the JAK/STAT pathway (Figure 2.3E). Finally, C1q bound to EAL and LAL increased the expression of NLRP12, a negative regulator of NF-κB and inflammasome activation (Figure 2.3F). When bound to LAL, C1q decreased the expression of NLRP3/NALP3 (also down-regulated by EAL alone by 2-fold with no further effect of C1q on this down-regulation, data not shown), one of the main components of the NLRP3 inflammasome, a cytosolic protein complex formed by the association of NLRP3, procaspase-1, and ASC (apoptosis-associated speck-like protein containing a CARD domain) that cleaves procaspase-1 to generate active caspase-1. At the same time, C1q bound to LAL increased the expression of POP1/ASC2 (Figure 2.3F), 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.

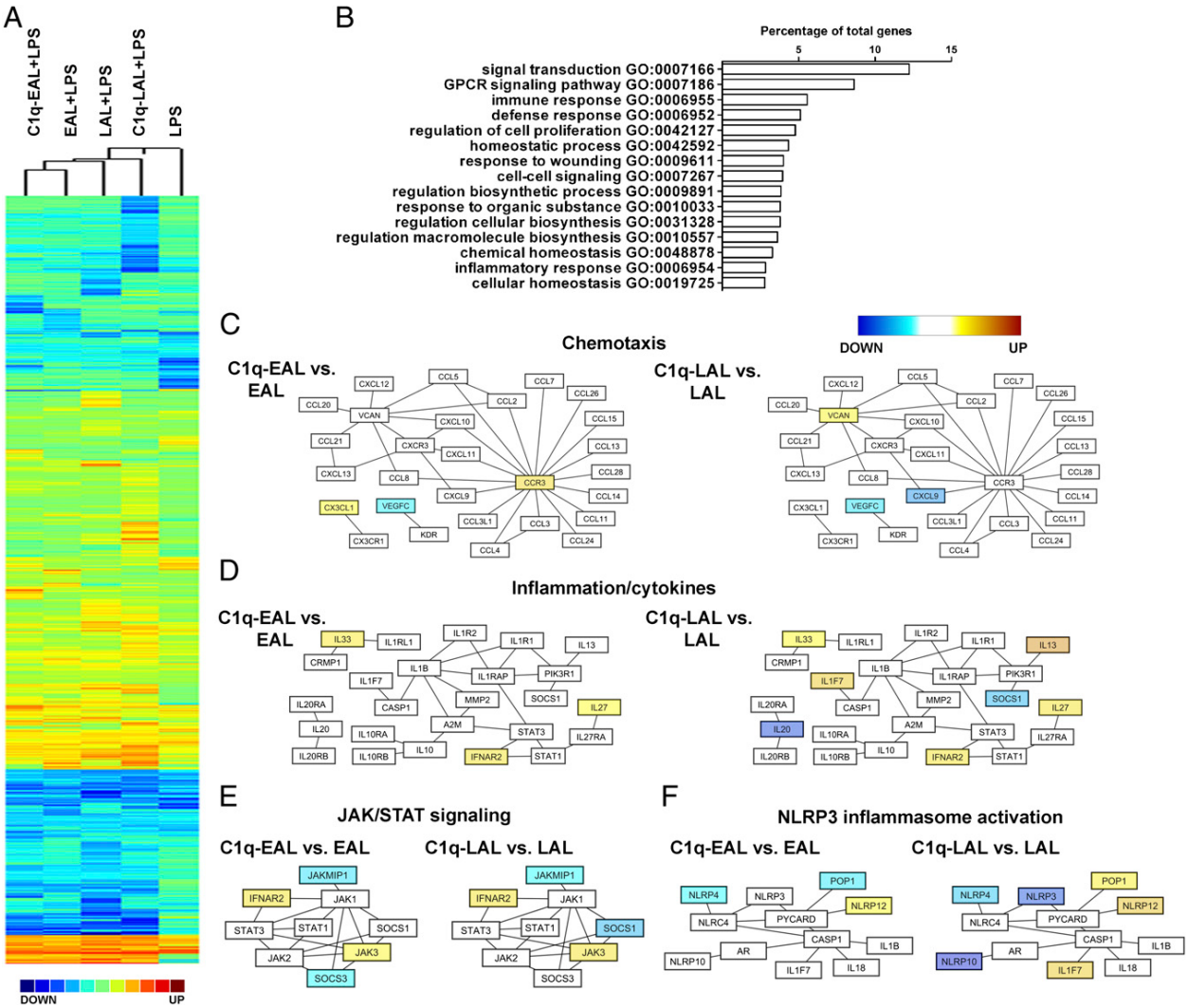


Figure 2.3 – Gene expression and main biological processes modulated by C1q in Mϕ during the uptake of AL. (A) Pearson correlation coefficient- based heat map (complete linkage method) representation of the $\log_2$fold-change (all conditions performed in triplicate) of Mϕ incubated with EAL, C1q–EAL, LAL, and C1q–LAL and then stimulated with LPS for 3 h over unstimulated Mϕ (B) GO-based functional annotation of genes modulated by C1q in Mϕ. 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 Mϕ. Node colors represent changes in gene expression in C1q– EAL versus EAL or C1q–LAL versus LAL, shown using a color gradient (blue, downregulated; white, not modulated; yellow, upregulated by C1q).

C1q increases STAT1 phosphorylation, type I IFN-s, IL-27 and IL-10 secretion by LPS-stimulated Mϕ during the uptake of AL

LPS stimulation of mouse macrophages induces IL-10 production through the sequential induction of type I IFN-s and IL-27 (Iyer et al., 2010). Since IL-27 was one of the most up-

regulated cytokine by C1q bound to EAL and LAL in Mϕ (about 4-fold-increase compared to unstimulated Mϕ and 2 fold-increase compared to EAL or LAL alone, Figure 2.3D) and IFN-A6 mRNA was slightly up-regulated by C1q in Mϕ (data not shown), we investigated if C1q modulates STAT1 phosphorylation (Tyr701), type I IFN-s, IL-27 and IL-10 expression in human macrophages. C1q bound to LAL (but not EAL) significantly increased levels of STAT1 phosphorylation after 30 min of LPS stimulation by flow cytometry (Figure 2.4A, B) and western blot (Figure 2.4 C, D)

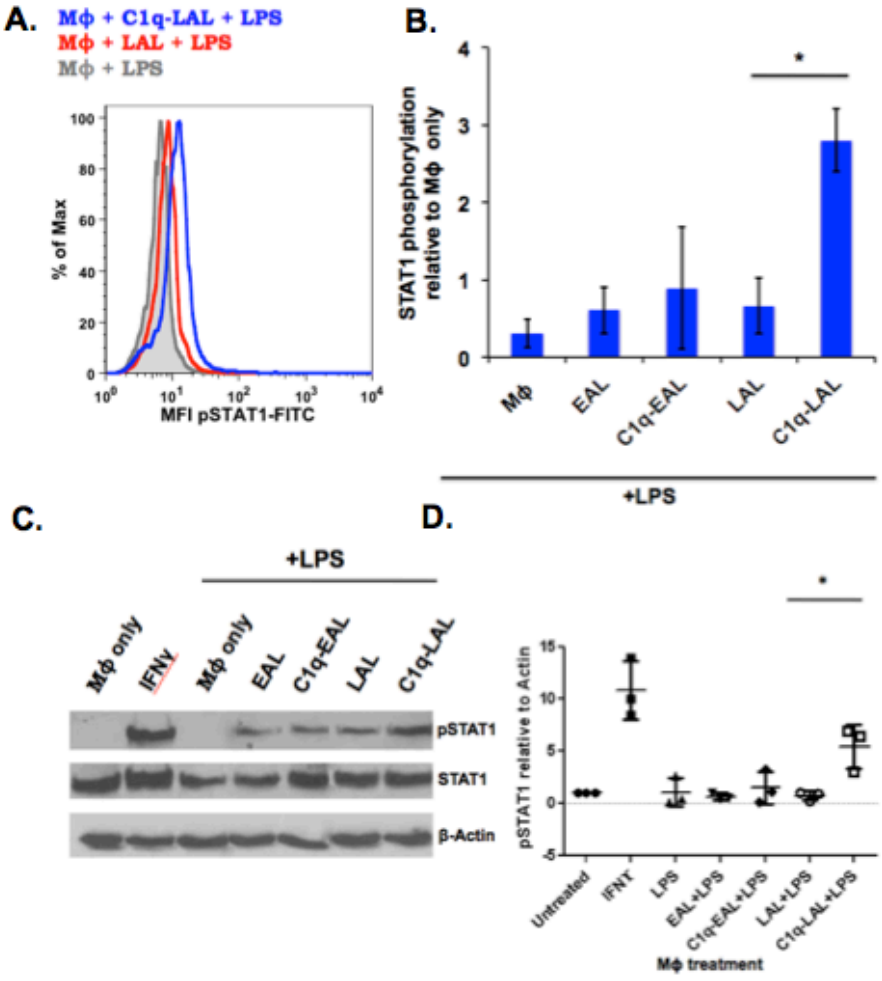


Figure 2.4 – C1q-bound LAL enhances STAT1 phosphorylation in Mϕ relative to LAL. Mϕ were incubated with AL for 60 minutes, washed and then stimulated with LPS for 30 minutes. (A) Representative plot of Mean fluorescence index (MFI) for phosphorylated STAT1 in LPS-stimulated Mϕ as measured by flow cytometry. (B) Average pSTAT1 MFI (3 independent experiments). (C) and (D) Cell extracts were probed for pSTAT1 (Tyr701), STAT1 protein and actin by western blot. (C) Representative blots from one of 3 independent experiments are shown. (D) pSTAT1 band intensity relative to actin quantification of 3 independent experiments. Results represent means ± s.d., * $p < 0.05$ by Student's *t*-test for LAL compared to C1q-LAL.

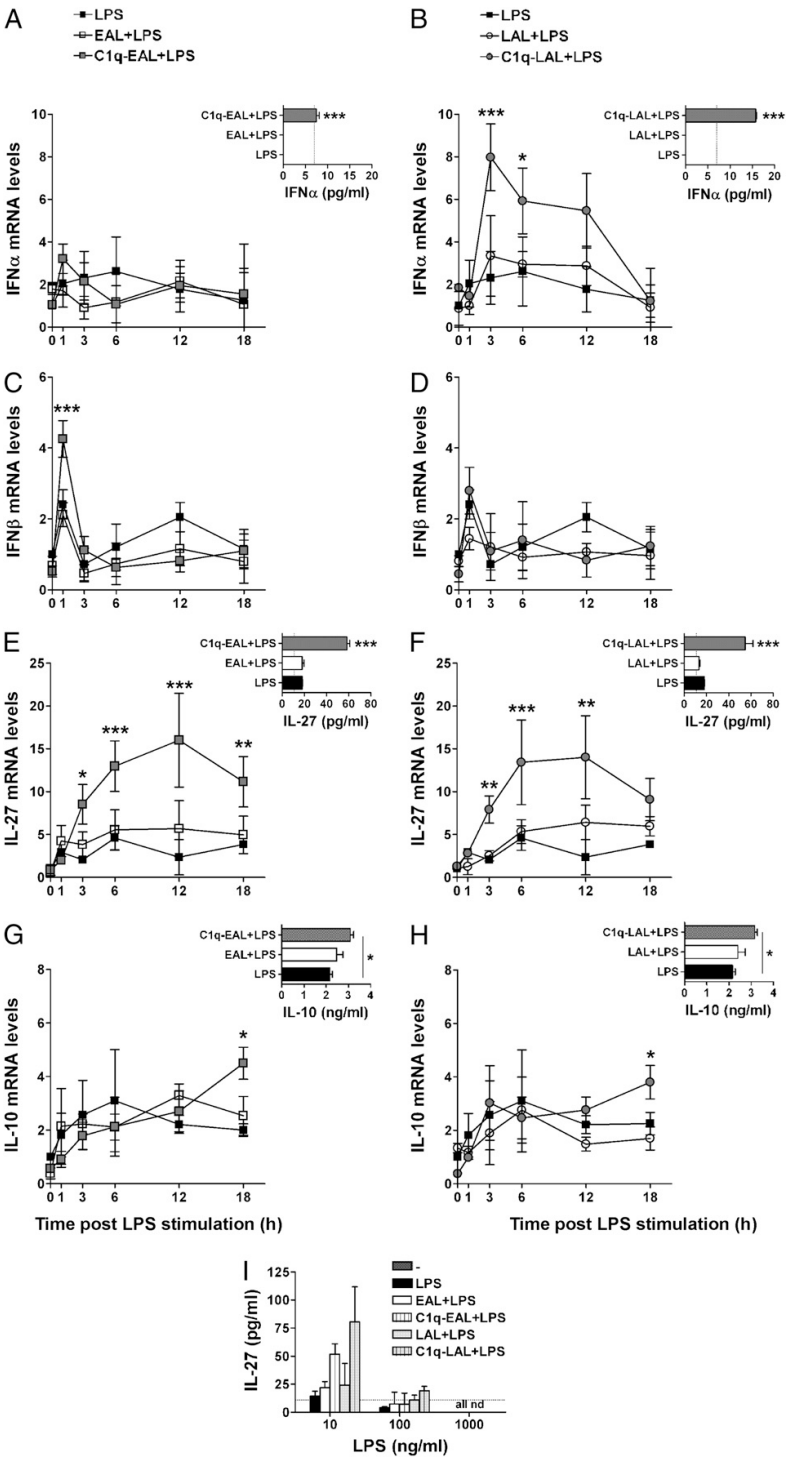


Figure 2.5 – Increase of type I IFN-s, IL-27, and IL-10 expression by C1q in LPS-stimulated Mϕ during the uptake of ALs. Mϕ were incubated with EALs (left panels) or LALs (right panels), preincubated or not with C1q, at a 5:1 ratio for 1 h and then stimulated with 10 ng/ml LPS (A–H) or 10–1000 ng/ml (I) in HL-1 for up to 18 h. Changes in mRNA levels for IFN--a (A, B), IFN--b (C, D), IL-27 (E, F), and IL-10 (G, H) were determined by qRT-PCR. Protein levels of IFN--a (A and B, insets) were detected 6 h after LPS stimulation and of IL-27 (E and F, insets; I) and IL-10 (G and H, insets) after 18 h of LPS stimulation. (A–H) LPS data (black squares) are identical between left and right panels (single LPS control experiment performed for both EALs and LALs simultaneously). Results represent means 6 SD (n = 2 to 3 different donors), two-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.

C1q transiently increased IFN-α and IFN-β mRNA levels (Figure 2.5A-D) in Mϕ, and the secretion of IFN-α by Mϕ was significantly ($p < 0.001$) increased by C1q bound to EAL or LAL after 6 h of LPS stimulation (Figure 2.5A and B, insets). C1q bound to EAL and LAL significantly increased IL-27 mRNA levels after 3, 6 and 12 h of LPS stimulation (Figure 3E and F) and the IL-27 protein levels were significantly ($p < 0.001$) increased by C1q-EAL and C1q-LAL after 18 h of LPS stimulation (Figure 2.5E and F, insets). Interestingly, 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 (Figure 2.5I). Finally, C1q bound to EAL and LAL significantly ($p < 0.05$) increased IL-10 mRNA levels in Mϕ after 18 h of LPS stimulation (Figure 2.5G and H). The protein levels of IL-10 were also increased by C1q after 18 h of LPS stimulation when compared to LPS ($p < 0.05$) and EAL or LAL (Figure 2.5G and H, insets).

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

We next investigated whether C1q modulates type I IFN-s, IL-27 and/or IL-10 in resting Mϕ (i.e. non LPS-stimulated). Mϕ were incubated with C1q-bound EAL or LAL 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). While C1q-bound to AL did not modulate expression of type I IFN-s or IL-10 at 3 h of incubation, C1q-bound to LAL significantly ($p < 0.01$) increased IL-27 mRNA levels compared to LAL alone (Figure 2.6A), although this 2-fold increase in IL-27 was lower than the induction observed in presence of LPS (Figure 2.5F).

Dying cells release numerous DAMPs that can activate TLR4, similarly to LPS (Kono and Rock, 2008). To model the more physiologic condition in which this release of DAMPs by EAL and LAL might affect the modulation of Mϕ response by C1q, Mϕ were incubated with C1q-bound EAL or LAL for 1 h and then incubated with EAL or LAL conditioned media for 3 h. While again at this time point, no induction of type I IFN-s or IL-10 was detectable, the presence of EAL conditioned media induced a 3-fold increase in IL-27 expression in Mϕ ingesting C1q-bound EAL compared to ingestion of EAL alone (Figure 2.6B). Moreover, in presence of LAL conditioned media, C1q-bound LAL induced a 6-fold increase in IL-27 expression in Mϕ compared to LAL alone (Figure2.6B), induction similar to what is observed in the presence of LPS without the conditioned media (Figure 2.5F). These results suggest that C1q sequentially induced type I IFN-s, IL-27 and IL-10 expression in LPS-stimulated Mϕ and may act in synergy with soluble factors released by dying cells to induce IL-27 in resting Mϕ.

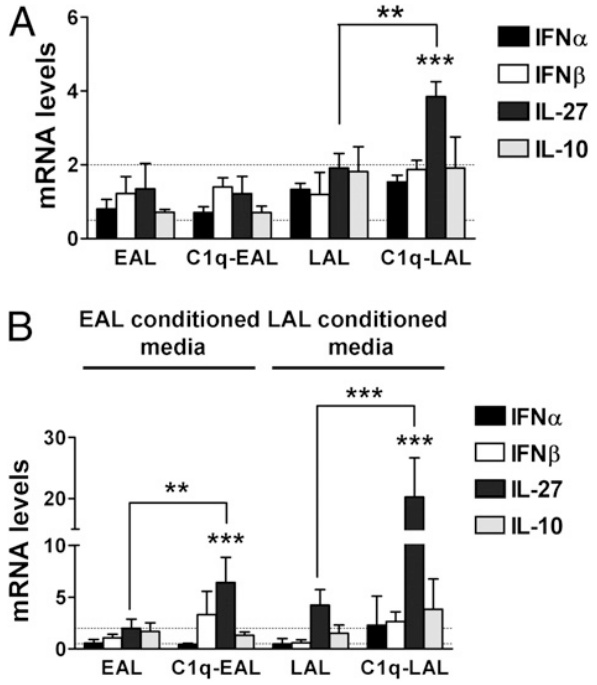


Figure 2.6 – Increased IL-27 expression by C1q in resting Mϕ during the uptake of AL. Mϕ were incubated with EAL or LAL, preincubated or not with C1q, at a 5:1 ratio for 1 h. Mϕ were then cultured in HL-1 for 3 h in absence of LPS (**A**) or in presence of EAL or LAL conditioned media (**B**). Changes in type I IFN-s, IL-27, and IL-10 were determined by qRT-PCR. Results represent means ± SD (n = 3different donors), two-way ANOVA. **p < 0.01, ***p < 0.001.

Co-incubation with C1q tails or neutralization of type I IFN-s suppresses IL-27 induction by C1q in LPS-stimulated Mϕ

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, Mϕ were incubated with C1q-bound EAL or LAL for 1 h in presence of increasing doses of C1q tails, which should compete with C1q bound to LAL to bind Mϕ without inducing C1q receptor clustering on Mϕ and thus no signaling. After washing to remove unbound targets and C1q tails, Mϕ were stimulated with LPS for 3 h and mRNA levels of IFN-α and IL-27 were determined by qRT-PCR (Figure2.7A and B). When Mϕ were incubated with C1q-bound EAL or LAL in presence of C1q tails, the C1q-induced expression of IFN-α and IL-27 is significantly decreased in a dose dependent manner by C1q tails (Figure 2.7A and B), suggesting a competition between C1q tails and C1q bound to AL to induce cytokines in Mϕ.

To determine the contribution of type I IFN-s in the induction of IL-27 and IL-10 by C1q in LPS-stimulated macrophages, Mϕ were incubated with C1q-bound EAL or LAL for 1 h and then stimulated with LPS for 18 h in presence of control mouse IgG or type I IFN-s or IL-27 neutralizing antibodies (Figure 2.7C-E). Incubation with anti-IFN-α antibodies, anti-IFN-β antibodies or both significantly decreased the IL-27 secretion induced by C1q bound to EAL to levels of EAL alone (Figure 2.7C). The induction of IL-27 in Mϕ by C1q bound to LAL was also significantly decreased by anti-IFN-α antibodies and/or anti-IFN-β antibodies, however the levels of IL-27 after neutralization of type I IFN-s remained significantly higher than IL-27 levels induced by LAL alone (Figure 2.7C). In addition, no change was observed in IL-10 levels after neutralization of type I IFN-s (Figure 2.7D) and neutralization of IL-27 slightly decreased

IL-10 protein levels but the differences did not reach statistical significance (Figure 2.7E). This suggests that induction of IL-27 by C1q is partially dependent on type I IFN-s in LPS-stimulated Mϕ while induction of IL-10 likely involves multiple pathways.

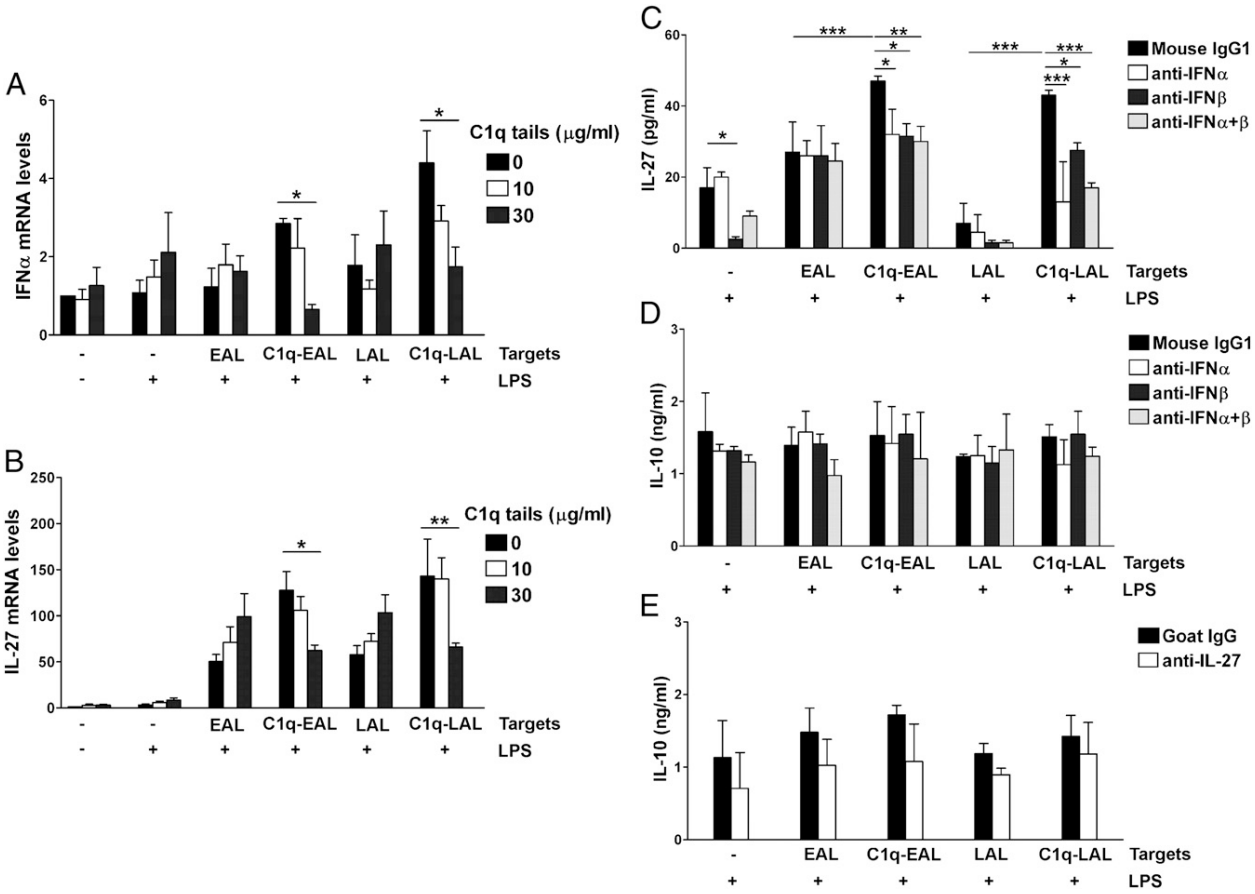


Figure 2.7 – Coincubation with C1q tails or inhibition of type I IFN-s reduces the induction of IL-27 by C1q in LPS-stimulated Mϕ. Mϕ were incubated with EALs, C1q–EALs, LALs, or C1q–LALs at a 5:1 ratio for 1 h (**A**, **B**) in presence of C1q tails and then stimulated with 10 ng/ml LPS for 3 h or (**C**–**E**) stimulated with LPS for 18 h in presence of 1 **m**g/ml control mouse IgG1 or neutralizing Abs against IFN-αand/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.

C1q suppresses NLRP3 inflammasome activation and IL-1β cleavage

Our microarray data suggest that EAL, C1q-EAL and C1q-LAL 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 (Figure 2.3). To

test this hypothesis, PKH26-prelabeled EAL and LAL (red) with or without bound C1q were added to Mϕ at a 5:1 ratio for 1 h. Mϕ 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 Mϕ were stained using a blue cell tracker (Figure 2.8A). About 30-40% of LPS-stimulated Mϕ and Mϕ that had ingested LAL showed cytoplasmic cleaved caspase-1 (Figure 2.8A and B). However, Mϕ that have ingested EAL, C1q-EAL or C1q-LAL showed almost no cleaved caspase-1 (the only cleaved caspase-1 signal detected in these Mϕ was associated with AL themselves) (Figure 2.8A and B). This decrease was slightly enhanced when counting only those Mϕ that had ingested C1q-LAL (3-fold decrease) vs. cleaved caspase-1 in Mϕ with and without evidence of ingested C1q-LAL (2-fold decrease) (Figure 2.7A and B). It is of note that while NLRP3 mRNA levels were down-regulated in Mϕ that have ingested EAL, C1q-EAL or C1q-LAL, the protein levels of NLRP3, as well as procaspase-1 and ASC, appear similar in all conditions at 6 h post LPS stimulation (Figure 2.8C).

Next, we examined whether the inhibition of caspase-1 cleavage by C1q resulted in decreased mature IL-1β levels released by macrophages. Mϕ were incubated with C1q bound to EAL or LAL 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 (Figure 2.8D), observations in accordance with gene expression data showing similar increased in IL-1β mRNA levels in all conditions. No mature IL-1β levels were detected in Mϕ supernatants in absence of ATP (Figure 2.8E). In presence of ATP, amount of mature IL-1β released by Mϕ was significantly ($p < 0.001$) increased after LPS stimulation and in LPS-stimulated Mϕ that have ingested LAL compared to unstimulated Mϕ (Figure 2.8E). Consistent with the observed

decreased in caspase-1 cleavage, Mϕ that had ingested EAL or C1q-bound EAL or LAL showed a significant decrease (50-60%, $p < 0.01$) in the amount of mature IL-1β released by Mϕ (Figure 2.8E). All together these results demonstrate that C1q inhibited cleavage of caspase-1 and subsequent IL-1β cleavage.

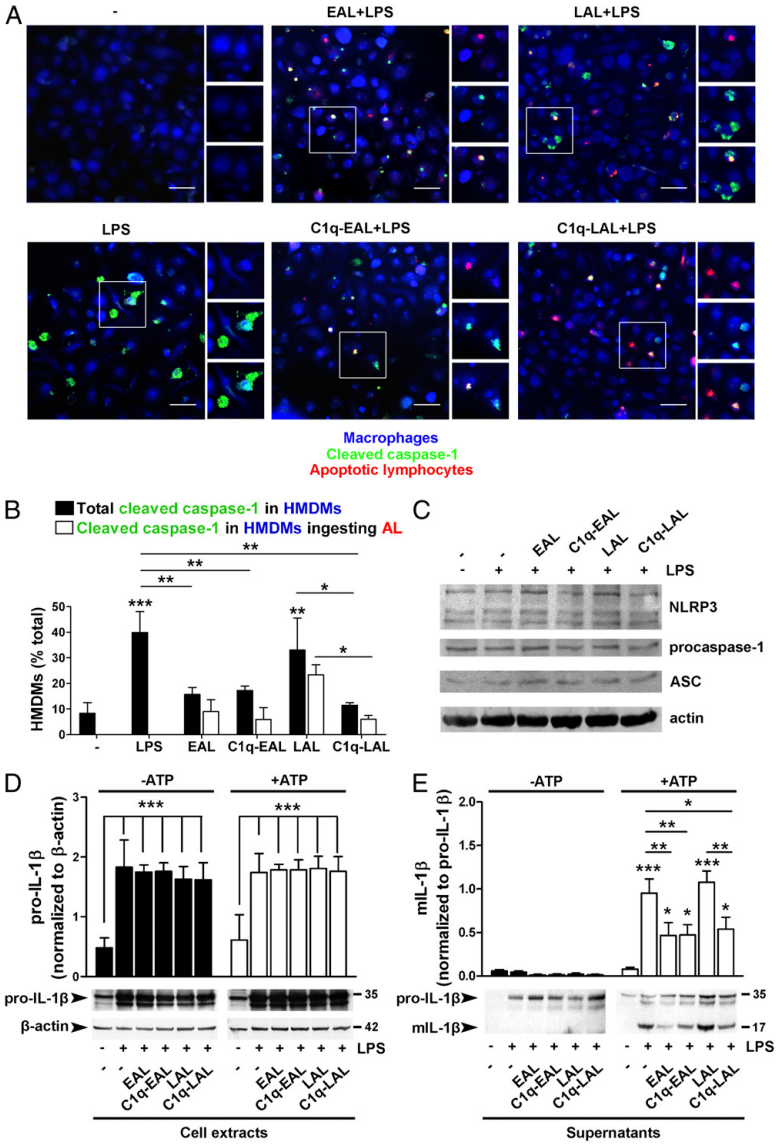


Figure 2.8 – C1q decreased procaspase-1 and pro–IL-1β cleavage in LPS-stimulated Mϕ. Mϕ were incubated with PKH26-labeled EAL and LALs, preincubated with C1q, at a 5:1 ratio for 1 h and then stimulated with 10 ng/ml LPS. (A and B) Mϕ were stimulated with LPS for 6 h. ATP (1 mM) was added 90 min before the end of the stimulation. Cleaved caspase-1 was detected by FITC fluorescent caspase-1 probes, and Mϕ were stained with a blue cell tracker. Representative merged micrographs of three independent experiments (from three different donors) are shown. Scale bar, 50 mm. 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 Mϕ. (C) NLRP3, procaspase-1, ASC, and actin expression in Mϕ cell extracts. Representative blots of two independent experiments are shown. (D and E) Levels of pro–IL-1β relative to b-actin levels (**D**, cell extracts) and mIL-1β relative to pro–IL-1β (**E**, supernatants) in Mϕ 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. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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 here show that in addition to enhancing phagocytosis of autologous apoptotic lymphocytes, C1q bound to apoptotic cell "cargo" significantly influences the responses of Mϕ in a more physiologic model than 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 (Figure 2.9).

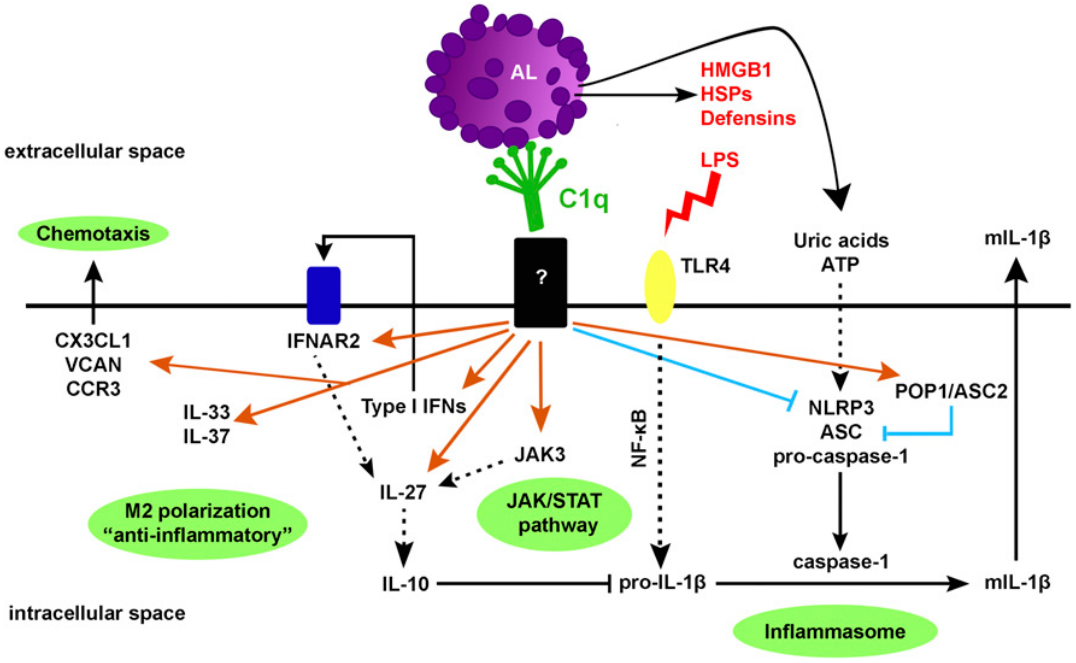


Figure 2.9 – Main biological pathways modulated by C1q in Mϕ. C1q increases the expression of type I IFN-s and IL-27, known to act sequentially to stimulate expression of IL-10 (also upregulated by C1q); IL-33, known to promote alternative activation of macrophages; IL-37, a potent natural suppressor of innate inflammatory responses; and JAK3, which may be involved in IL-27 upregulation. C1q also suppresses procaspase-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.

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 (Gullstrand et al., 2009;Fraser et al., 2009). Our data showed that C1q, at physiological concentrations, binds directly, and to the same extent, to primary human EAL and LAL, suggesting differences between primary and transformed apoptotic cells in this regard. The fact that C1q binds strongly and stably to EAL in our system supports a central role of C1q in the rapid removal of apoptotic cells to avoid auto-immunity.

C1q enhanced the uptake of EAL and LAL by Mϕ, in accordance with numerous previous reports showing an enhancement of uptake of apoptotic cells bound to C1q by different subsets of phagocytes (Fraser et al., 2009;Fraser et al., 2010;Gullstrand et al., 2009;Nauta et al., 2004). While 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 EAL and LAL indeed profoundly affected the Mϕ response to LPS, this effect being sometimes dependent on the stage of the apoptotic cell (early vs. late). It is likely that C1q engaged multiple receptors on Mϕ in addition to the engagement of other phagocytic receptors, such as Mer, SR-A or CD36 (Erwig and Henson, 2008;Galvan et al., 2012a;Galvan et al., 2014) by the apoptotic cells themselves. This would trigger a complex signaling cascade, which involves STAT1 phosphorylation but remains to be fully characterized, leading to the modulation of the different pathways identified in this study. C1q bound to EAL and LAL regulated the expression of several cytokines in Mϕ. 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 (Biswas and Mantovani, 2010;Kurowska-Stolarska et al., 2009), which is also induced by C1q bound to LAL. In addition, C1q bound to

LAL increased the expression of IL-37 (IL1F7), a natural suppressor of innate inflammatory responses (Nold et al., 2010). In previous studies, C1q has been shown to enhance IL-10 production after LPS stimulation (Fraser et al., 2006;Fraser et al., 2009;Fraser et al., 2010). Among myeloid cells, macrophages are the main source of IL-10, which is produced in response to TLR stimulation to limit and resolve inflammation (Mosser and Zhang, 2008). Recently, it has been shown that induction of IL-10 in LPS-stimulated murine macrophages results from the sequential induction of type I IFN-s and IL-27 (Iyer et al., 2010). The data presented here show that C1q sequentially induces STAT1 phosphorylation, type I IFN-s, 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 doses of LPS, suggesting that the immunoregulatory effect of C1q 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 which 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 IFN-s and IL-27, consistent with the reported binding of C1q to apoptotic cells via the globular heads, and thus orienting the "tail" domain to interact with the macrophage and induce cytokine expression in Mϕ. Finally, neutralization of type I IFN-s only partially decreased the induction of IL-27 and neutralization of IL-27 moderately affected the induction of IL-10. In addition, IL-27 was also up-regulated by C1q in resting Mϕ while no increase in type I IFN-s was observed in these cells. These results suggest that type I IFN-s participate in the induction of IL-27 and possibly IL-10 in Mϕ stimulated with low dose of LPS, but that these pathways likely involve other partners in resting Mϕ that are still to be identified.

C1q has been shown to inhibit IFN--α production by plasmacytoid dendritic cells (pDCs) in response to stimulation by SLE immune complexes *in vitro* and C1q deficiency has been associated with defective regulation of IFN--α production by pDCs in SLE patients (Santer et al., 2010). This apparent opposite effect of C1q on regulation of type I IFN-s production can be due to differences in the source of type I IFN-s between these two systems (Mϕ and pDCs), the transient and very local induction of type I IFN-s in our system vs. a systemic dysregulation effect with immune complexes and pDCs and/or the differences in the stimulus (apoptotic cells/PAMPs/DAMPs vs. immune complexes). These observations reinforce the premise that depending on the context, the stimulus and the cell type it interacts with, the outcome of the C1q response may be widely different. These differences must be considered in the development of therapeutics targeting these pathways.

Importantly, our data provides the first demonstration that C1q bound to "self-cargo" directly inhibits inflammasome activation, cleavage of caspase-1 and subsequent IL-1β processing in Mϕ. Interestingly, late apoptotic cells that exhibit decreased membrane integrity ("secondary necrosis") release numerous DAMPs that can trigger inflammasome activation (ATP and uric acids) and/or activate TLR4 such as high mobility group box 1 (HMGB1), heat shock proteins (HSPs) and defensins (Kono and Rock, 2008). By directly regulating inflammasome activation, C1q may prevent excessive and dysregulated inflammasome activation induced by these DAMPs (Figure 7). The mechanism by which C1q is modulating inflammasome activation still under investigation but one possible hypothesis is that C1q bound to LAL increases negative regulators of inflammasome activation such as POP1/ASC2 mRNA levels. POP1 (which is not encoded in the mouse genome (Stehlik and Dorfleutner, 2007)) has been shown to bind ASC and modulate NF-κB activation and procaspase-1 cleavage (Stehlik et

al., 2003) in human macrophages. Others have reported that inhibition of inflammasome activation by autocrine IL-10 is dependent on signaling through JAK3 (Kim et al., 2004), 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 IFN-s (Guarda et al., 2011), JAK3 and IL-10. Finally, C1q bound to EAL and LAL increased the expression of NLRP12, an important inhibitor of inflammatory gene expression in human myeloid cells through suppression of NF-κB activation (Williams et al., 2005). 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 apoptotic lymphocytes and significantly modulated gene expression profile and inflammasome activity of Mϕ (Figure 2.9). Importantly, the predominant effect of C1q on Mϕ inflammatory responses was observed when C1q is bound to late apoptotic cells since 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.

Chapter 3: Complement Protein C1q Bound to Apoptotic Cells Suppresses Human Macrophage and Dendritic-Cell Mediated Th17 and Th1 T cell Subset Proliferation

Abstract

A complete genetic deficiency of the complement protein C1q results in systemic lupus erythematous (SLE) with nearly 100% penetrance in humans, but the molecular mechanisms responsible for this association have not yet been fully determined. C1q opsonizes apoptotic cells (AC) for enhanced ingestion by phagocytes such as macrophages (Mϕ) and immature dendritic cells (DC), avoiding the extracellular release of inflammatory damage-associated molecular patterns (DAMPS) upon loss of the membrane integrity of the dying cell. It has been previously shown that human monocyte-derived Mϕ and DC ingesting autologous C1q-bound late apoptotic lymphocytes (LAL) (C1q-polarized Mϕ and C1q-polarized DC) enhance the production of anti-inflammatory cytokines and reduce pro-inflammatory cytokines relative to Mϕ or DC ingesting LAL alone. Here, I show that C1q-polarized Mϕ have elevated PD-L1 and PD-L2 and suppressed surface CD40 and C1q-polarized DC have higher surface PD-L2 and less CD86 relative to Mϕ or DC ingesting LAL alone, respectively. In a mixed-leukocyte reaction (MLR) C1q-polarized Mϕ reduced allogeneic and autologous Th17 and Th1 subset proliferation, and demonstrated a trend towards increased Treg proliferation relative to Mϕ ingesting LAL alone. Moreover, relative to DC ingesting AC in the absence of C1q, C1q-polarized DC decreased autologous Th17 and Th1 proliferation. These data demonstrate that a functional consequence of C1q-polarized Mϕ is the regulation of T effector cell activation, thereby "sculpting" the adaptive immune system to avoid autoimmunity while clearing dying cells. Importantly, these studies

identify novel target pathways for therapeutic intervention in SLE and other autoimmune diseases.

Introduction

C1q is the recognition component of the classical complement pathway with a prominent role in initiating complement-dependent killing of invading microbes and clearance of immune complexes. It is well-established that a genetic deficiency of C1q in humans leads to the development of systemic lupus erythematous (SLE) with nearly 100% penetrance (Walport et al., 1998). In mice, complete genetic C1q deficiency (or reduced macrophage C1q production) results in autoantibody production and murine lupus nephritis on certain strain backgrounds (Botto et al., 1998;Mitchell et al., 2002;Miura-Shimura et al., 2002), consistent with the function of this protein as a regulator of inflammation and autoimmunity. Moreover, in murine Mϕ C1q increases Mer tyrosine kinase (MerTK), a critical receptor tyrosine kinase for the uptake of apoptotic cells (Galvan et al., 2012a;Zizzo et al., 2012). Given that C1q binds to apoptotic cells via its globular head domains (Korb and Ahearn, 1997), it is hypothesized that C1q functions in this context by opsonizing apoptotic cells for more rapid removal by phagocytes before they undergo secondary necrosis (reviewed in (Botto and Walport, 2002;Elkon and Santer, 2012)), which avoids the release of inflammatory damage-associated molecular patterns (DAMPs) and dangerous alarmins (Abdulahad et al., 2010).

However, additional molecular mechanisms responsible for the C1q-mediated protection against autoimmunity in humans are beginning to be elucidated. In humans, C1q-bound to immune complexes (ICs) markedly shifted IC binding to monocytes and away from pDCs thereby reducing the expression of the majority of IFN--response genes induced by ICs (Santer et al., 2012). The Tenner lab and others have demonstrated that C1q modulates phagocyte production of cytokines, enhancing anti-inflammatory and reducing proinflammatory cytokines (Lu et al., 2008;Fraser et al., 2009;Castellano et al., 2004b). In addition, Lu and colleagues (Teh

et al., 2011) demonstrated that primary human monocytes differentiated to dendritic cells in the presence of immobilized C1q showed reduced induction of allogeneic Th1 and Th17 cells. Previous studies have shown that human monocyte-derived immature dendritic cells (DC) ingesting C1q-bound autologous apoptotic cells (AC) (C1q-polarized DC) increase secretion of anti-inflammatory cytokines such as IL-10 and reduce pro-inflammatory cytokines such as TNF-α (Fraser et al., 2009). However, many of these initial studies had either evaluated the effect of C1q on the ingestion of apoptotic cells generated from transformed cell lines (Fraser et al., 2009), or assessed C1q-cytokine responses and signaling in primary human monocytes or Mϕ using plate bound presentation of C1q (Teh et al., 2011;Galvan et al., 2012a;Fraser et al., 2007). Recently, a model was developed in which primary human Mϕ ingest more physiologically relevant autologous late apoptotic lymphocytes (LAL) to which C1q is bound. In this system, macrophages ingesting C1q-bound LAL promote the successive gene expression and production of Type 1 IFN- and subsequently the anti-inflammatory cytokines IL-27 and IL-10, as well as decreasing inflammasome activity and secretion of mature IL-1β (Benoit et al., 2012). These data suggest that C1q is crucial not only for the effective clearance of dying cells, but also for suppressing the inflammatory environment in a human autologous system.

Regulation of the adaptive immune response is critical for the avoidance of autoimmunity. For instance, T cells can contribute to SLE pathogenesis causing B cells to produce pathogenic autoantibodies in the inductive phase as well as producing proinflammatory cytokines during the effector phase (Tsokos, 2011). Polarized macrophages, programmed by PAMPs, DAMPs and the microenvironment, secrete different cytokines and chemokines and express different surface markers, which together activate or suppress different populations of T cells, which then impacts the immune response. For example, the costimulatory ligand CD40 is

known to enhance Th1 responses (Peng et al., 1996) and CD86 can trigger Th1 (Lichtenegger et al., 2012) and Th17 (Kang et al., 2007) cell skewing. Th17 cells produce IL-17, which causes follicular Th cells to promote autoantibody-producing B cells (Ding et al., 2013) and contribute to pathology (Crispin and Tsokos, 2010) in human and mouse models of SLE. In contrast, IL-27 is a direct and indirect negative regulator of the Th17 cell lineage (Mascanfroni et al., 2013;Diveu et al., 2009;Kido et al., 2011;Sugiyama et al., 2008). Increases in IL-27 have been shown to be due at least in part to Mϕ increase in Type I IFN-s acting back on the Mϕ in an autocrine fashion (Iyer et al., 2010;Guarda et al., 2011). Thus, the sequential increase in Type 1 IFN-, IL-27, and IL-10 gene expression and protein production by Mϕ ingesting C1q-bound LAL (Benoit et al., 2012) is consistent with the hypothesis that C1q could attenuate T-cell mediated autoimmunity by increasing levels of these cytokines. Additionally, IL-27 acting on dendritic cells has been shown to upregulate CD39, an ectoenzyme that decreases the extracellular concentration of ATP and thus attenuates ATP-dependent activation of the NLRP3 inflammasome and ultimately suppresses DC-mediated Th17 proliferation (Mascanfroni et al., 2013). Both PD-L1 whose expression is induced by IL-27 (Karakhanova et al., 2011) on human monocyte-derived DCs and PD-L2, elevated on alternatively activated mouse macrophages (Huber et al., 2010), are known to suppress antigen-dependent T effector cell activation via interaction with the T cell inhibitory receptor PD-1 (Fife et al., 2009;Latchman et al., 2001).

Regulatory T cells play an essential role in maintaining immune homeostasis and preventing autoimmunity (Sakaguchi et al., 2008). Defects in Treg development, maintenance or function have been associated with SLE (Cretney et al., 2013). Surfactant protein A (SP-A), a lung tissue-specific defense collagen with similar structure and function to C1q, dramatically increases the proliferation of the Treg lineage in a mixed-leukocyte reaction (MLR) (Mukherjee

et al., 2012). More recently, a novel type of regulatory T cell, CD8+Foxp3+ (CD8+ Tregs), has been identified that completely prevented mortality due to graft-versus-host disease (GVHD) after allogeneic stem cell transplantation in mice in the absence of CD4+ Tregs (Beres et al., 2012). Thus, these CD8+Foxp3+ cells may reduce inflammatory T cell responses and promote tolerance.

In this study, I discovered that human Mϕ and dendritic cells (DC) ingesting autologous C1q-bound LAL (C1q-polarized Mϕ and DC) suppressed the induction of allogeneic and autologous Th17 and Th1 cell proliferation. In addition to the previously reported enhanced production of IL-27 and IL-10, C1q-polarized human Mϕ have decreased levels of CD40 and increased levels of PD-L1 and PD-L2 on the cell surface. Furthermore, primary human C1q-polarized DC upregulated PD-L2, downregulated CD86, and enhanced IL-27 expression relative to DC ingesting LAL alone. Taken together, these data identify a novel pathway by which C1q interaction with APCs modulates the adaptive immune response, can prevent the initiation and propagation phases of autoimmunity, suppress human autoimmune inflammation, and potentially promote tolerance.

Materials and Methods

Media and reagents

RPMI 1640, penicillin/streptomycin, trypsin-EDTA and L-Glutamine were purchased from Invitrogen, while X-VIVO-15 medium was purchased from Lonza and defined fetal bovine serum (FBS) was from HyClone. Recombinant human (rh) M-CSF, IL-2, IL-7, IL-4 GM-CSF, IL-1β, IL-6 and TGF--β1 were from PeproTech. Human serum albumin (HSA) used for monocyte elutriation was obtained from Talecris Biotherapeutics. Ultra-pure LPS was from List Biological Laboratories Inc. R848 (TLR 7/8 agonist) was purchased from Enzo Life Sciences. CFSE was from Molecular Probes. C1q was isolated from plasma-derived normal human serum by ion-exchange chromatography, followed by size exclusion chromatography according to Tenner et al. (Tenner et al., 1981) and modified as described (Young, Jr. et al., 1991). All C1q preparations were homogeneous (determined by SDS-PAGE and Coomassie staining) and had less than 0.03 EU/ml endotoxin by Limulus Amoebocyte Lysate clot assay (Lonza).

Human peripheral blood leukocyte isolation and culture

All blood samples were collected into CPDA1 at the UCI Institute for Clinical and Translational Science in accordance with guidelines and approval of the University of California, Irvine (UCI) Institutional Review Board. Human peripheral blood lymphocytes and monocytes were isolated from peripheral blood mononuclear cells (PBMCs) by countercurrent elutriation as previously described (Benoit et al., 2012), with >80% CD3+ cells in the lymphocyte fraction and 90 – 98% CD11b + in the monocyte fraction. Human monocyte derived macrophages (Mϕ) were generated from monocytes by culture at 2 x 10^6 cells/ml for 6 – 8 days in RPMI1640, 10% FBS, 2 mM L-Glutamine and 1% penicillin/streptomycin (complete media) containing 25 ng/ml rhM-

CSF with addition of fresh media + rhM-CSF at day 3. Human monocyte derived dendritic cells (DC) were generated from monocytes by culturing at 2 x 10^6 cells/ml for 6 – 8 days in complete media containing 25 ng/ml rhGM-CSF + 25 ng/ml rhIL-4 with addition of fresh media + rhGM-CSF + rhIL-4 at day 3. For MLR and surface expression experiments, the adherent Mϕ or DC were harvested by washing twice with 1x HBSS and incubating with non-enzymatic CellStripper (CellGro) for 20 – 30 min. Autologous lymphocytes were maintained for 6 – 8 days in complete media containing 50 U/ml rhIL-2 and then γ-irradiated (10 Gy) and maintained overnight in media without FBS to generate apoptotic lymphocytes (LAL) (40 – 60% Annexin V+/PI+) (Benoit et al., 2012). For every experiment, apoptosis (apoptosis detection kit from BioVision), C1q binding to LAL and Mϕ phenotype was assessed by flow cytometry as previously described (Benoit et al., 2012).

Phagocytosis of LAL

Mϕ or DC ingestion of LAL in 96-well round-bottom plates (Nunc) was evaluated as previously described (Benoit et al., 2012). Briefly, LAL (in some cases prelabeled with PKH26 (Sigma)), pre-coated or not with C1q were incubated with 100 µl containing 3 x 10^4 Mϕ or DC at a 5:1 ratio for 1 hr in phagocytosis buffer (RPMI1640, 25 mM HEPES and 5 mM $MgCl_2$). The Mϕ or DC were then washed twice in 1x HBSS to clear away the uningested LAL and C1q-LAL. For phagocytosis quantification, cells were washed, harvested with Trypsin/EDTA (Gibco), stained with anti CD11b-FITC antibodies (Invitrogen) for flow cytometry and scored for PKH26+ CD11b+ double positive cells. The percentage of Mϕ and DCs phagocytosing at least one LAL within one hour ranged from 48 - 64% and 27 - 44%, respectively, by CD11b+PKH26+ double staining.

Detection of surface marker expression

To evaluate CD40, PD-L1, PD-L2, CD86 and CD39 expression, Mϕ or DC were cultured for 24 hr after the ingestion of apoptotic cells (as described above) in 1 ml X-VIVO-15 in the presence or absence of 10 ng/ml LPS where indicated. Mϕ or DC were harvested with 1 ml Cellstripper (Mediatech), washed in HBSS containing 3% (w/v) BSA and 0.05% (w/v) NaN_3 and then incubated for 30 min with either α-CD40-PE (Invitrogen), α-PD-L1-PE or α-PD-L2-PE (eBioscience) and α-CD11b-FITC or CD86-FITC, α-CD39-APC and α-CD11b-PE (Biolegend) according the manufacturer's instructions. Cells were washed and analyzed using a FACS Calibur (BD Biosciences). All CD11b+ cells were included in the analysis.

Reverse transcription and quantitative real-time PCR

At 4.5, 5 or 6.5 hours, 2.5 x 10^5 Mϕ or DC were washed twice in HBSS and then lysed for 5 min in buffer RA1 (GE Healthcare Life Sciences) according to the manufacturer instructions. Total RNA was harvested using the illustra RNAspin Mini Kit (GE Healthcare Life Sciences). cDNA synthesis was carried out with 200 ng of total RNA and SuperScript® Reverse Transcriptase (Invitrogen) according to manufacturer instructions. Quantitative PCR was performed as previously described (1). Probes used were: IL-27F 5'-CGGAGGGAGTTCACAGTCAG-3' and IL-27R 5'-GAAGGTCAGGGAAACATCAGG-3'. The fold-change (FC) was determined as follows: $FC = 2^{-\Delta Ct}$, where $\Delta Ct = (Ct_{IL\text{-}27} - Ct_{GAPDH})$. Ct values were defined as the number of cycles required for the fluorescent signal to cross the threshold, i.e., exceed background level (2).

Mixed-Leukocyte Reaction (MLR)

T cells used for MLR experiments were purified from PBMCs to ≥ 95% CD3+ using EasySep™ negative selection T cell isolation kit (StemCell Technologies). Allogeneic T cells were used on the same day as isolated, while autologous T cells were maintained for 7 days (during the differentiation of monocytes into Mϕ or DC) in complete media containing 50 U/ml rhIL-7. In some experiments, CD45RO-PE and α-CD45RA-PerCP-Cy5.5 (eBioscience or Tonbo) were used to characterize the resulting populations of T cells. Fifty microliters (1×10^5 cells) of allogeneic or autologous primary human T cells, pre-labeled with 5uM CFSE, were added to 200ul of the Mϕ or DC (a 3:1 ratio) 24 hours after the phagocytes had ingested LAL or C1q-bound LAL (and treated with or without 10ng/ml LPS or 5 uM R848 as indicated). In some experiments, recombinant human IL-1β (20 ng/ml), IL-6 (30 ng/ml) and TGF--β1 (2.25 ng/ml) were added to cultures at the start of the MLR. After 5 and 6 days, the non-adherent cells were removed (routinely, 12 replicate wells were pooled per condition), restimulated with 50 ng/ml PMA (Sigma) and 50 ug/ml ionomycin (EMD Millipore) in the presence of 3 ug/ml brefeldin-A (eBioscience) for 6 hours. After washing, cells were first stained for surface markers CD8-Pacific Blue (BioLegend) and α-CD4-PE, and subsequently for intracellular markers with α-Foxp3-APC, α-IL-17-PE-Cy7 and IFN-γ-APC-Cy7 (all from eBioscience). Intracellular antigens were detected by blocking with 2% normal mouse serum and staining with the Foxp3 staining buffer set from eBioscience (following the manufacturer's instructions). In initial experiments, T cells were incubated with IL-23, IL-12p70 or IL-2 and stimulated anti-CD3 and anti-CD28 for 3 days to generate positive controls for the detection of Th17 (CD4+IL-17+), Th1 (CD4+IFN-γ+), Treg (CD4+Foxp3+) phenotypes, respectively. For MLR flow cytometry experiments, we used

the following gating strategy. Using an equivalent number of cells per sample, a sample of T cells which had been pre-stained with propidium iodide (PI) and subsequently washed was then fixed, permeabilized and processed identically to all other samples. A gate was drawn around the PI -negative cells (live cells) and the live gate was expressed as FSC/SSC and applied to the other samples. In subsequent experiments, this FSC/SSC "live" gate was applied to each sample. The live cells were then gated on α-CD4-PE or α-CD8-Pacific Blue and MFI of Foxp3, IL-17 or IFN-γ and CSFE was assessed by flow cytometry on a BD LSRII. Flow cytometric data were analyzed using FlowJo software (Tree Star) or FlowLogic software (eBioscience). The influence of C1q on T cell lineage was assessed by calculating the ratio (fold change) of % proliferating specific T cell subsets in the MLR containing Mϕ or DC that had ingested C1q-LAL vs. Mϕ or DC that ingested LAL in the absence of C1q. The two tailed student t-test in Prism version 6 (GraphPad) was used for statistical analysis. For each analysis, error bars are SD, and *, ** or *** indicates the p value, referring to the difference between the mean fold change value of each subset and a theoretical mean of 1.0 by "one sample T test" (two-tailed).

Results

C1q-polarized Mϕ express elevated levels of programmed death ligands 1 and 2 and reduced levels of CD40 relative to macrophages ingesting LAL.

To begin to explore the functional consequences of C1q interaction with Mϕ, the induction of PD-L1 and PD-L2 molecules in Mϕ 24 hr after ingesting apoptotic cells was evaluated. To provide a mild inflammatory stimulus to the Mϕ that could be present in the area of dying cells, a low level of the TLR4 agonist LPS (10 ng/ml) was added immediately after the removal of uningested apoptotic cells. Late apoptotic lymphocytes (LAL) were used as these cells are beginning to lose their membrane integrity, and thus to release proinflammatory molecules (such as HMGB1, a TLR ligand (Yu et al., 2006)). While all Mϕ that had ingested LAL significantly upregulated PD-L1 expression, Mϕ that had ingested LAL to which C1q was bound, i.e. C1q-polarized Mϕ, expressed significantly more PD-L1 relative to macrophages that had ingested LAL without C1q both in the presence and absence of LPS (Figure 3.1 A, B). C1q-polarized Mϕ also significantly increased levels of PD-L2 relative to macrophages ingesting LAL in the presence of LPS (Figure 3.1 C, D). The magnitude of the increase of PD-L2 expression on C1q-polarized Mϕ in the presence of added LPS was less than that of PD-L1, but was still statistically significant. C1q also promoted a slight trend toward increased expression of the surface regulatory molecule CD39 (data not shown).

To determine whether C1q-polarized Mϕ influence the expression of co-stimulatory molecules which could have an impact on antigen-dependent T cell activation, we assessed the levels of surface CD40 in these Mϕ. In the absence of LPS, CD40 expression on Mϕ increased 3 fold upon ingestion of LAL, whereas addition of C1q bound LAL dampened this response (Figure 3.1 E, F). A similar effect was noted with the addition of LPS to cultures, in which C1q

bound LAL limited the up-regulation of CD40 on Mϕ after ingestion (Figure 3.1 E, F). In contrast, C1q did not influence Mϕ expression of the co-stimulatory ligand CD86 relative to LAL in either the presence or absence of LPS (data not shown).

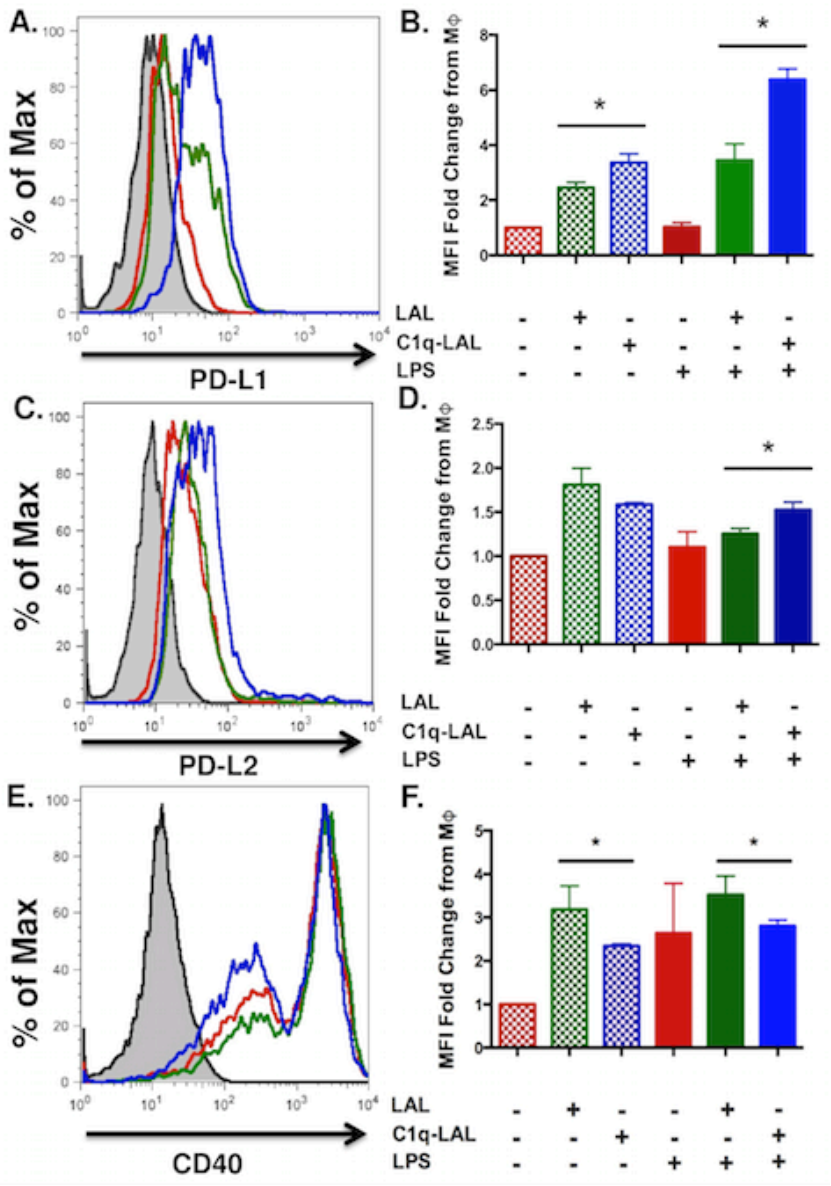


Figure 3.1 – Macrophages ingesting C1q-bound apoptotic cells are polarized towards an anti-inflammatory phenotype. C1q-coated autologous apoptotic lymphocytes (C1q-LAL) (blue) or LAL only (green) were added to M-CSF monocyte-derived macrophages (Mϕ) (A – F) in a 5:1 ratio for 1h, after which the uningested apoptotic cells were washed away. Mϕ were then stimulated or not with 10 ng/ml LPS in X-VIVO-15 for 24h. The adherent cells were harvested, stained with CD11b-FITC and anti PD-L1-PE (A,B), anti PD-L2-PE (C,D) or anti CD40-PE (E,F), and read by flow cytometry. Controls were Mϕ without LAL (red). Grey shading (A, C, E) indicates PE-isotype control. Representative FACS plots gated on CD11b positive cells are shown for samples with LPS added (A,C, E). Average fold change MFI (FC) relative to Mϕ only (B,D,F) for 3 – 4 independent experiments. *$p < 0.05$ by Students T-test. Error bars are S.D.

C1q-polarized dendritic cells express elevated levels of programmed death ligand 2 and reduced levels of CD86 relative to macrophages ingesting LAL.

In order to define the functional phenotype of C1q-polarized DC, we evaluated surface marker expression of DC ingesting C1q-bound LAL. In the presence of LPS, C1q triggered significantly more PD-L2 expression relative to DC ingesting LAL alone (Figure 3.2 A, B). Interestingly, however, C1q-polarized DC did not exhibit levels of PD-L1 that differed from DC ingesting LAL without C1q (data not shown). C1q-polarized DC exhibited slightly elevated (but statistically significant) surface expression levels of the anti-inflammatory ectoenzyme CD39 relative to DC ingesting LAL alone (data not shown). In addition, while DC ingesting LAL without C1q had a pronounced induction of CD86, C1q-polarized DC attenuated this LAL-triggered enhancement of CD86 expression in the presence of LPS (Figure 3.2 C, D). However, C1q did not affect the levels of CD40 on DC relative to LAL without C1q (data not shown).

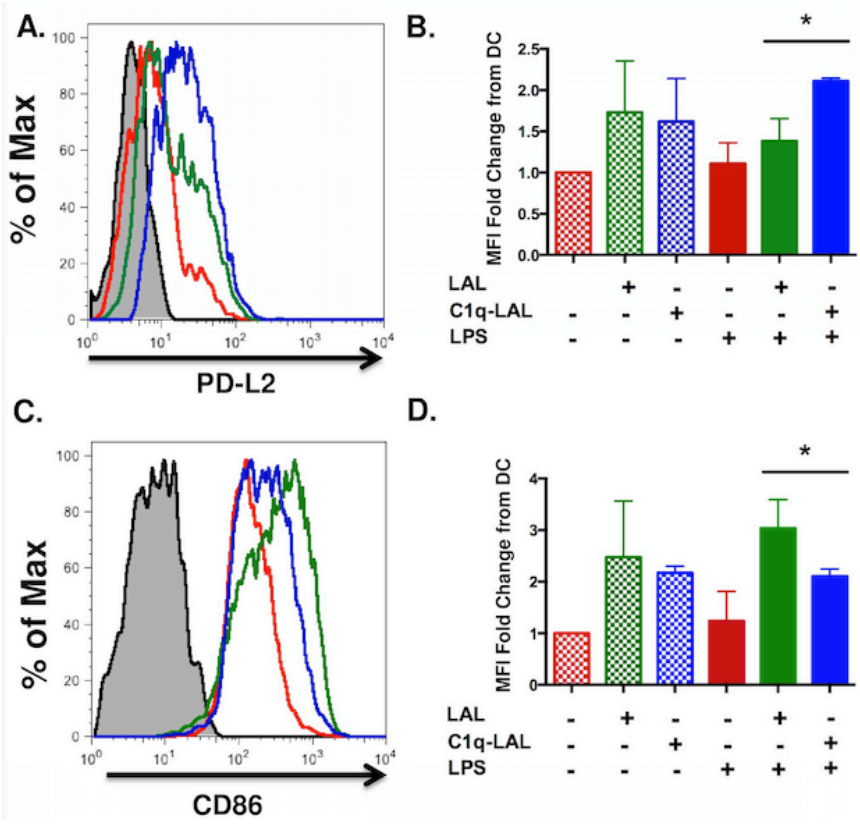


Figure 3.2 – Immature dendritic cells ingesting C1q-bound apoptotic cells are polarized towards an anti-inflammatory phenotype. C1q-coated autologous apoptotic lymphocytes (C1q-LAL) (blue) or LAL only (green) were added to GM-CSF + IL-4 monocyte-derived dendritic cells (DC) (A – D) in a 5:1 ratio for 1h, after which the uningested apoptotic cells were washed away. DC were then stimulated or not with 10 ng/ml LPS in X-VIVO-15 for 24h. The adherent cells were harvested, stained with CD11b-FITC and anti PD-L2-PE (A, B) or CD11b-PE and anti CD86-FITC (C, D), and read by flow cytometry. Controls were DC without LAL (red). Grey shading (A, C) indicates PE-isotype control. Representative FACS plots gated on CD11b positive cells are shown for samples with LPS added (A, C). Average fold change (FC) MFI relative to DC only (B, D) for 4 – 5 independent experiments. *$p < 0.05$ by two-tailed Students T-test. Error bars are S.D.

C1q-polarized Mϕ suppress the proliferation of human allogeneic inflammatory T cells and trend toward enhanced proliferation of human allogeneic regulatory T cells.

Since PD-L1 and PD-L2 binding to programmed-death 1 (PD-1) on the surface of the T cell results in inhibition of T cell activation, and CD40 acts as a co-stimulatory ligand known to activate T cells during autoimmunity (reviewed in (Chatzigeorgiou et al., 2009)), we assessed the ability of primary human Mϕ that ingested LAL with C1q bound to their surface to direct the differential stimulation of primary human T cell subsets in a mixed leukocyte reaction (MLR).

After the ingestion of LAL, Mϕ were treated with a low level of LPS (10 ng/ml) as described above for 24 h, followed by addition of purified allogeneic T cells to the Mϕ for 6 days. In this entirely human primary cell system with its inherent diversity of individual donor responses, C1q-polarized Mϕ consistently suppressed the proliferation of the inflammatory T cell subsets. Specifically, the increase in proliferation of CD4+IL-17+ (Th17) cells triggered by Mϕ that had ingested LAL was suppressed by LAL with bound C1q by an average of 29% ($n=5$, $p<0.02$) relative to Mϕ that had ingested LAL in the absence of C1q (Figure 3.3 A-C, J, K) (Table 3.1). Importantly, this suppression resulted in a level of proliferation that was often back to the baseline level of proliferation seen with T cells co-cultured with Mϕ that had not ingested apoptotic cells.

Similarly, C1q-polarized Mϕ decreased the allogeneic proliferation of IFN-γ producing inflammatory subsets. CSFE dilution of CD4+IFN-γ+ (Th1) cells was decreased by an average of 32% ($p<0.03$) (Figure 3.3 D – F, J, K) (Table 3.1). Proliferation of CD8+IFN-γ+ cells was also decreased by C1q-polarized Mϕ by an average of 22% though this trend was not statistically significant ($p<0.1$) (Figure 3.3 G – I, J, K) (Table 3.1).

C1q-polarized Mϕ also exhibited a trend towards enhanced proliferation of CD4+Foxp3+ (Treg) cells in 3 of 5 sets of donors (1.43 to 2.68-fold increase, $p=0.36$, $n=5$) and CD8+Foxp3+ cells in 4 of 5 sets of donors (1.17 to 3.61-fold increase, $p=0.29$, $n=5$) relative to LAL after 6 days of co-culture with allogeneic T cells (Figure 3.3 J, K) (Table 3.1). These data suggest that the microenvironment generated by the C1q-polarized Mϕ may favor proliferation of these regulatory cells, but that other factors also influence these subpopulations.

In the absence of extrinsic TLR ligand, the effect of C1q on Mϕ-mediated allogeneic T cell subset proliferation was not statistically significant for any subset (data not shown),

consistent with previous reports demonstrating that low levels of LPS provide a level of proinflammatory stimulation which is then reduced by C1q, such as seen with its effect on the modulation of cytokines and surface marker expression (Benoit et al., 2012;Fraser et al., 2009;Castellano et al., 2007;Fraser et al., 2006).

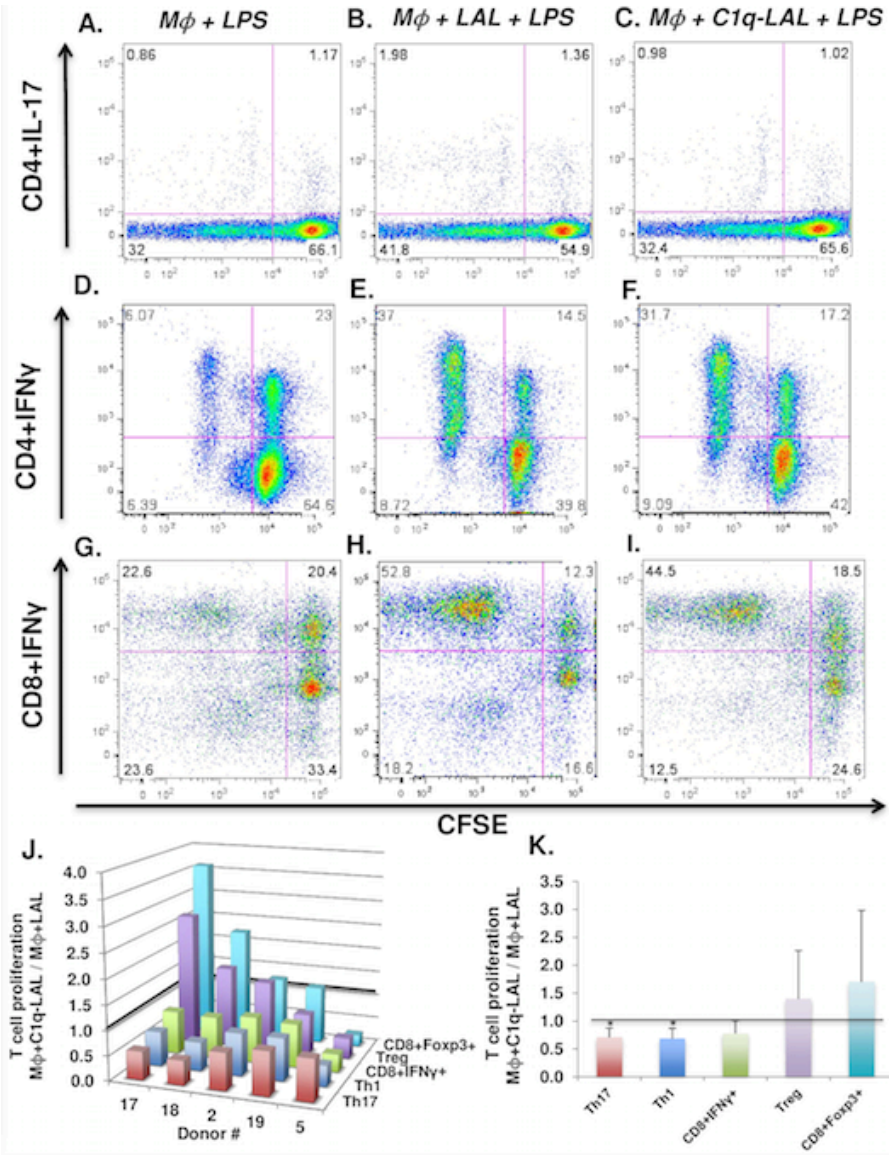


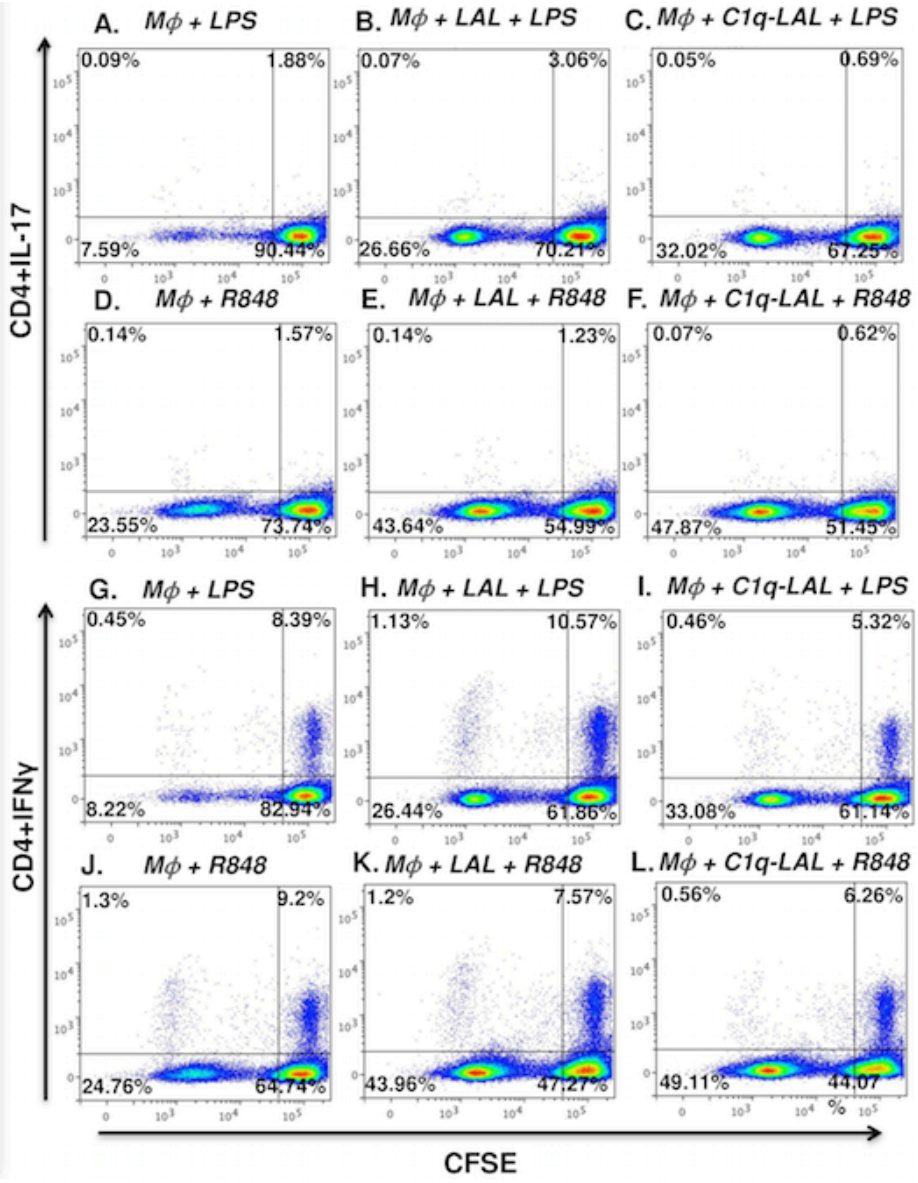
Figure 3.3 – C1q suppresses macrophage-mediated allogeneic T cell proliferation of inflammatory T cell subsets. CFSE-stained allogeneic primary human T cells (95% CD3+) were added (3:1) 24 hours after LPS treatment of Mϕ that had ingested LAL (B, E, and H) or C1q-bound LAL (C, F and I) or without apoptotic cells (A, D and G) as described in Figure 1. After 6 days, the non-adherent cells were removed, restimulated with 50 ng/ml PMA and 50 ug/ml ionomycin in the presence of 3 ug/ml brefeldin-A for 6 hours, then stained with α-CD4-PE, α-CD8-Pacific Blue, IL-17-PE-Cy7, and IFN-γ-APC-Cy7, and assessed by flow cytometry on a BD LSRII. Using Flow-Logic, events were gated on α-CD4-PE (A – F) or α-CD8-Pacific Blue (G – I) and MFI of IL-17-PE-Cy7 (A – C), IFN-γ-APC-Cy7 (G – I) and CSFE dilution (% proliferating and non-proliferating cells) depicted. Data are representative of 5 independent experiments. Percent of total events is indicated in each quadrant. (J) The ratio of Mϕ+C1q-LAL / Mϕ+LAL for the percent proliferating Th17, Th1, CD8+IFN-γ+, Treg, and CD8+Foxp3+, cells for 5 donors is shown for Mϕ stimulated LPS. Darkened line at 1.0 equals no difference between for Mϕ+C1q-LAL / Mϕ+LAL) for the percent proliferating subsets. (K) Average fold change Mϕ+C1q-LAL / Mϕ+LAL for the percent proliferating subsets of 5 donors. [1.0 would indicate no difference between Mϕ+C1q-LAL and Mϕ+LAL.] Error bars are SD, and * indicates $p<0.03$, referring to the difference between the mean fold change value of each subset and a theoretical mean of 1.0 by "one sample T test" (two-tailed).

C1q-polarized Mϕ suppress proliferation of autologous inflammatory T cells

To investigate whether C1q may reduce inflammatory T cell subset activation and enhance regulatory T cell lineages in situations of autoimmune inflammation such as may be involved in SLE or similar diseases, we modified our experimental design to use autologous rather than allogeneic T cells in our T cell activation assay. To maintain T cells from the same donor during Mϕ differentiation, purified CD3+ cells were maintained in IL-7 for 7 days, which results in nearly 100% CD45RA+ cells (data not shown), consistent with previous reports (Vollger and Uittenbogaart, 1993;Rathmell et al., 2001;Bretschneider et al., 2013). Under these conditions, C1q-polarized Mϕ suppressed inflammatory Th17 autologous T cell proliferation in all donors with the average decrease of nearly 50% ($n = 13$, $p<0.0001$), (Figure 3.4 A – C, M,N) (Table 3.1). Similarly, C1q-polarized Mϕ initiated less Th1 proliferation relative to macrophages ingesting LAL in all donors (average of 39% decrease, $p <0.0001$), (Figure 3.4 G – I, M,N) (Table 3.1). However, C1q did not have a consistent effect on CD8+INFγ+ cell proliferation (Figure 3.4 M,N) (Table 3.1).

Similar to the allogeneic MLR, in the absence of extrinsic TLR ligand, C1q did not instigate a statistically significant effect on Mϕ-mediated autologous T cell subset proliferation for any subset (data not shown), consistent with earlier studies demonstrating that C1q acts as a "check" on low level stimulation of inflammatory cytokines and surface marker expression (Benoit et al., 2012;Fraser et al., 2009;Castellano et al., 2007;Fraser et al., 2006). Finally, there was no consistent effect on Foxp3+ subset proliferation, likely in part due to the very limited presence of these subpopulations in the T cells cultured for 7 days in IL-7 while the Mϕ differentiation was induced.

To pursue whether this in vitro culturing of T cells with IL-7 may influence the generality of our findings, we differentiated monocytes to Mϕ and added freshly isolated T cells from blood of same donor drawn a week later than that for the monocytes. In three donors we found that Mϕ ingesting C1q-LAL suppress Th17 and Th1 proliferation relative to Mϕ ingesting LAL by an average of 25% and 34%, respectively. Similar to the autologous MLR containing T cells that had been maintained in IL-7, there was no consistent C1q effect on either CD4+ or CD8+ Treg proliferation.



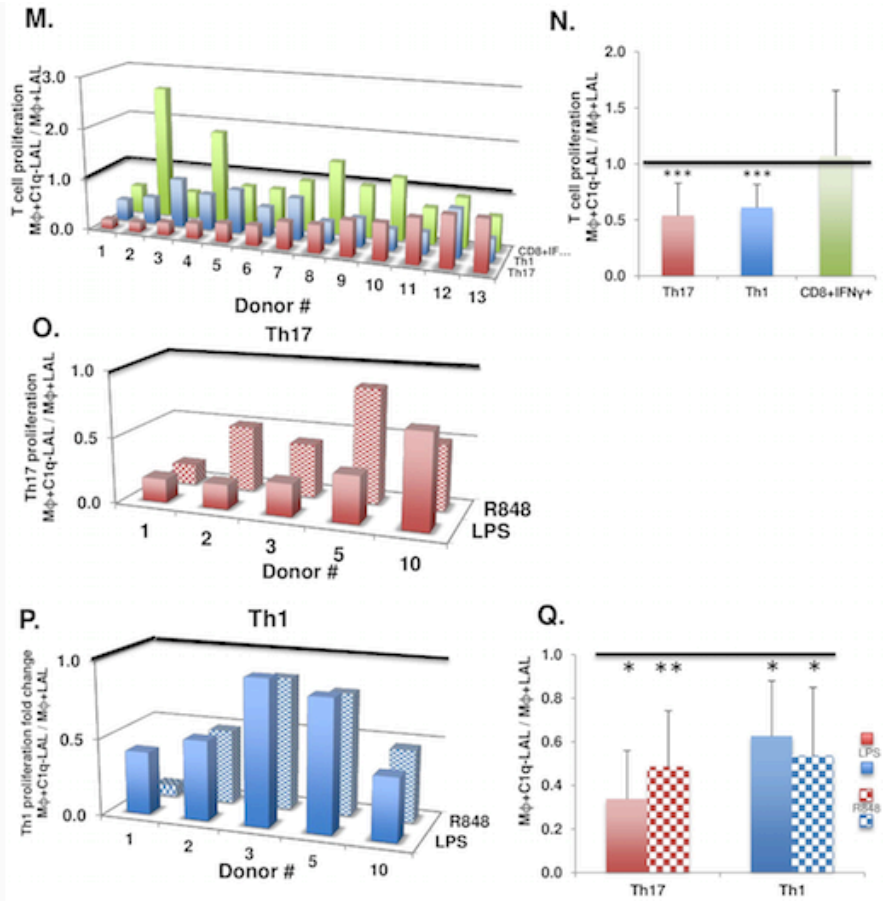


Figure 3.4 – C1q suppresses macrophage-mediated autologous T cell proliferation of inflammatory T cell subsets in the presence of exogenous TLR4 and TLR7/8 ligands. Autologous primary human T cells (95% CD3+) cultured in IL-7 for 8 days were prelabeled with CSFE and added 24 hours after 10 ng/ml LPS (TLR4 agonist) (A-C,G-I) or 5 uM R848 (TLR 7/8 agonist) (D-F,J-L) treatment of Mϕ that had ingested LAL (B and H or E and K) or C1q-bound LAL (C and I or F and L) or without apoptotic cells (A, D, G and J) as described in Figure 1. After 6 days, the non-adherent cells were removed, treated as described in Figure 3.3 and assessed by flow cytometry on a BD LSRII. Using Flow-Logic, cells were gated on α-CD4-PE and MFI of IL-17-PE-Cy7 (A – F), IFN-γ-APC-Cy7 (G – L) and CSFE dilution (% proliferating and non-proliferating cells) depicted. Percent of total events is indicated in each quadrant. (M) The ratio of Mϕ+C1q-LAL vs. Mϕ+LAL for percent proliferating Th17, Th1 and CD8+IFN-γ+ cells for 13 independent experiments. Darkened line at 1.0 equals no difference between for Mϕ+C1q-LAL / Mϕ+LAL) for the percent proliferating subsets. (N) Average ratio of the percent proliferating subsets in the presence of Mϕ+C1q-LAL relative to Mϕ+LAL for the 13 donors. (O, P) The ratio of the percent proliferating Th17 (O) or Th1 (P) cells in the presence of Mϕ+C1q-LAL relative to Mϕ+LAL is shown for 5 donors stimulated with 10 ng/ml LPS (blue) or 5 uM R848 (red). Darkened line at 1.0 equals no difference between Mϕ+C1q-LAL and Mϕ+LAL) for the percent proliferating subsets. (Q) Average ratio of Mϕ+C1q-LAL vs. Mϕ+LAL for 5 donors for the percent proliferating subsets in the presence of either LPS (blue) or R848 (red) added to cultures. Error bars are SD, *** indicates $p<0.0001$, ** indicates $p<.01$, * indicates $p<0.03$, by two tailed t-test for the difference between the proliferating subset average value and 1.0 (1 indicates no difference between Mϕ+C1q-LAL and Mϕ+LAL) for each subset.

C1q-polarized Mϕ suppress inflammatory T cell subsets in an autologous MLR when TLR 7/8 agonist R848 is added to the Mϕ prior to the MLR

A number of studies have demonstrated a clear role for TLR7 in SLE pathogenesis (Shen et al., 2010;Kelley et al., 2007). To evaluate whether the presence of another TLR ligand would affect the influence of C1q-polarized Mϕ on autologous T cell subset induction and proliferation in the same manner as the TLR4 ligand LPS, we added R848 (TLR 7/8 ligand) instead of LPS to Mϕ after ingesting LAL or C1q-bound LAL. C1q-polarized Mϕ treated with R848 decreased Th17 proliferation by an average of 51% ($p<0.02$), similar to LPS treated macrophages in parallel wells (Figure 3.4D – F, O, Q). Additionally, C1q-polarized Mϕ consistently suppressed Th1 proliferation by an average of 46% in the presence of R848, similar to LPS treated macrophages from the same donor (Figure 3.4J – L, P, Q). In contrast, C1q bound to LAL did not consistently affect CD8+IFN-γ+ T cell proliferation when R848 was added to the macrophages, similar to the lack of effect of C1q on this subset in the presence of the low level TLR4 signal (data not shown).

Allogeneic Th17 proliferation				
Donor #	LAL-Prolif. %	C1q-LAL-Prolif. %	C1q-LAL/LAL	% Reduction
2	20.70	18.20	0.88	12
5	5.52	4.63	0.84	16
17	0.30	0.17	0.57	43
18	1.98	0.98	0.49	51
19	0.04	0.03	0.75	25

Allogeneic Th1 proliferation				
Donor #	LAL-Prolif. %	C1q-LAL-Prolif. %	C1q-LAL/LAL	% Reduction
2	37.00	31.70	0.86	14
5	10.64	4.44	0.42	58
17	52.40	36.90	0.70	30
18	8.18	4.74	0.58	42
19	9.83	8.42	0.86	14

Autologous Th17 proliferation				
Donor #	LAL-Prolif. %	C1q-LAL-Prolif. %	C1q-LAL/LAL	% Reduction
1	0.28	0.05	0.18	82
2	0.16	0.03	0.19	81
3	0.89	0.22	0.25	75
4	1.49	0.44	0.30	70
5	0.64	0.23	0.36	64
6	0.72	0.28	0.39	61
7	1.91	1.01	0.53	47
8	1.31	0.71	0.54	46
9	0.29	0.20	0.69	31
10	0.07	0.05	0.71	29
11	0.23	0.20	0.87	13
12	2.23	2.20	0.99	1
13	1.43	1.42	0.99	1

Autologous Th1 proliferation				
Donor #	LAL-Prolif. %	C1q-LAL-Prolif. %	C1q-LAL/LAL	% Reduction
1	0.49	0.20	0.41	59
2	0.83	0.43	0.52	48
3	1.19	1.12	0.94	6
4	1.40	0.98	0.70	30
5	4.66	3.98	0.85	15
6	2.36	1.34	0.57	43
7	10.82	8.79	0.81	19
8	9.00	3.75	0.42	58
9	1.13	0.62	0.55	45
10	1.13	0.46	0.41	59
11	0.60	0.25	0.42	58
12	19.14	17.68	0.92	8
13	1.94	0.83	0.43	57

Table 3.1 –Spread of raw proliferation subset data for allogeneic and autologous MLR. Values arranged by donor number.

Effect of exogenous cytokines on C1q-mediated suppression of Th17 proliferation

In these autologous MLR experiments, no exogenous Th17-promoting cytokines were added and accordingly, the number of Th17 cells was generally low. Thus, we performed an MLR in the presence of recombinant human IL-1β (20 ng/ml), IL-6 (30 ng/ml) and TGF--β1 (2.25 ng/ml) in the cultures to promote Th17 development (19). We found that in the presence of these exogenous cytokines, C1q-LAL promoted an average of approximately 1.72-fold greater suppression (average of 88% in the presence and 51% suppression in the absence of exogenous cytokines) of Th17 proliferation than C1q-LAL in the absence of exogenous cytokines (n = 2, Figure 3.5).

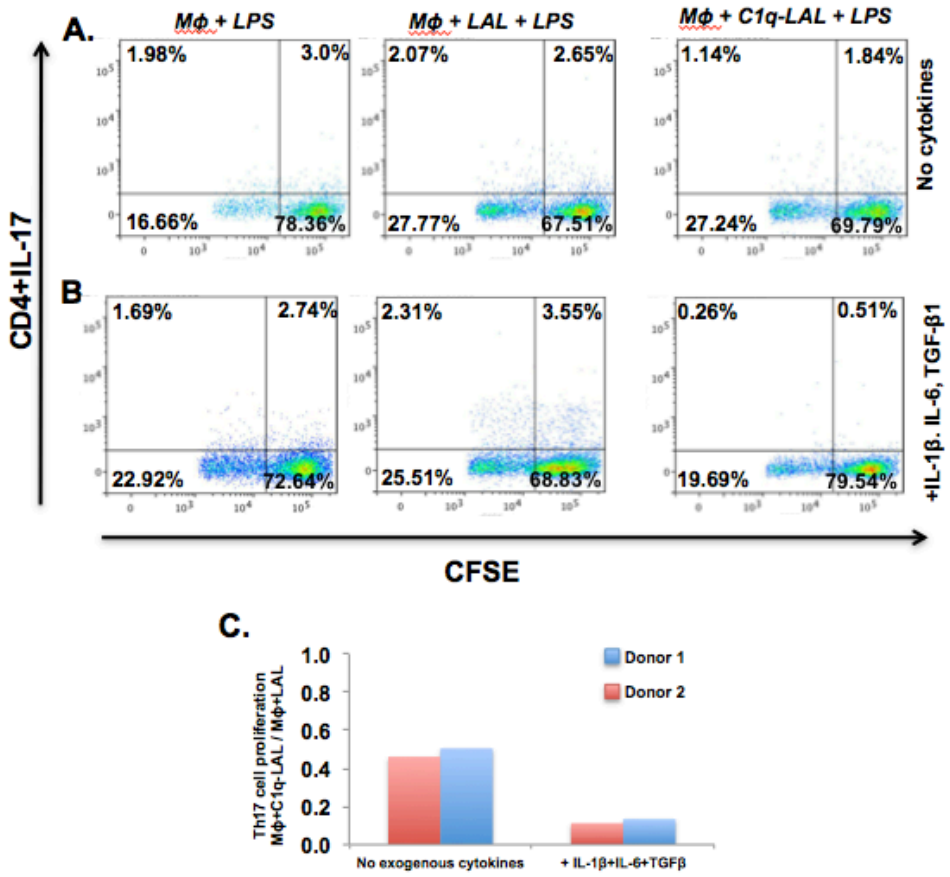


Figure 3.5 – C1q enhances suppression of Th17 proliferation in the presence of exogenous cytokines. A. MLR performed in the absence of exogenous cytokines using cells from the same donor as in (B). B. Effect of adding exogenous recombinant human IL-1β (20 ng/ml), IL-6 (30 ng/ml) and TGF--β1 (2.25 ng/ml) to cultures at the start of the MLR. The MLR and data collection were otherwise performed identically to Figure 3.3. Quantification of C1q suppression of Th17 proliferation (Mϕ+C1q-LAL / Mϕ+LAL) in the presence and absence of exogenous human IL-1β, IL-6 and TGF--β1.

C1q-polarized Mϕ conditioned media suppresses inflammatory T cell subsets in an autologous MLR.

To evaluate whether the effect of C1q-polarized Mϕ on autologous T cell subset proliferation and induction is due at least in part to soluble factors (such as increased IL-27 or IL-10) versus surface-bound factors (such as increased PD-L1, PD-L2 or reduced CD40), conditioned media (supernatants) from 24 hr LPS-treated C1q-polarized Mϕ was added to untreated, M-CSF-derived Mϕ plus autologous T cells. Similar to the effect observed with C1q-polarized Mϕ in parallel wells from the same donor, conditioned media from C1q-polarized Mϕ reduced the percentage of proliferating Th17 cells relative to Mϕ ingesting LAL alone in all

cases by 43%; n=5, p = 0.05), (Figure 3.6A – F, M – O). Additionally, relative to conditioned media from Mϕ ingesting LAL alone, conditioned media from C1q-polarized Mϕ reduced the percentage of proliferating Th1 cells by an average of 38% (n=5; $p<0.04$) to a similar extent as the polarized Mϕ themselves.

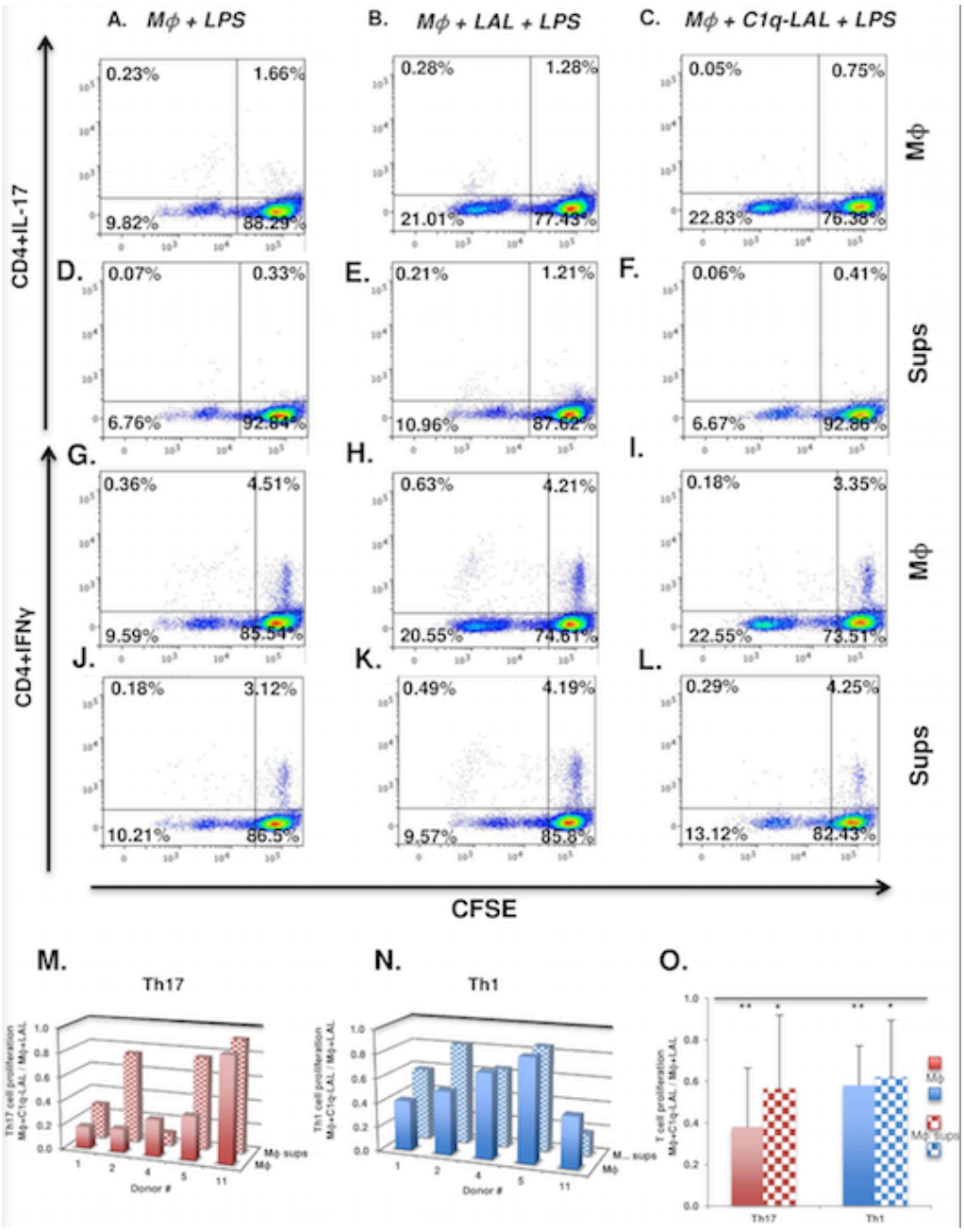


Figure 3.6 – Culture supernatants from C1q-polarized macrophage suppress autologous T cell proliferation of inflammatory T cell subsets. Mϕ were treated as described in Figure 4 (A – C and G – I) or with 24-hour conditioned media from LPS treated C1q-polarized Mϕ (F and L), Mϕ ingesting LAL without C1q (E and K) or Mϕ alone (D and J). CFSE-stained autologous primary human T cells (95% CD3+) were added (3:1). After 6 days, the non-adherent cells were removed, restimulated, stained and fluorescence assessed by flow cytometry on a BD LSRII as described in Figure 3.3. Using Flow-Logic, cells were gated on α-CD4-PE and MFI of IL-17-PE-Cy7 (A – F) and IFN-γ-APC-Cy7 (G – L) and CFSE dilution (% proliferating and non-proliferating cells) is depicted. Data are representative of 5 independent experiments for each subset. Percent of total events is indicated in each quadrant. (M and N) The ratio of the percent proliferating Th17 (M) or Th1 (N) cells for 5 donors is shown for Mϕ in the presence of Mϕ+C1q-LAL conditioned media relative to Mϕ+LAL conditioned media (Mϕ sups) or Mϕ+C1q-LAL relative to Mϕ+LAL (Mϕ). Darkened line at 1.0 equals no difference between for Mϕ+C1q-LAL / Mϕ+LAL) for the percent proliferating subsets. (O) Average ratio of Mϕ+C1q-LAL conditioned media relative to Mϕ+LAL conditioned media (Mϕ sups) or Mϕ+C1q-LAL relative to Mϕ+LAL (Mϕ) for 5 donors for the percent proliferating subsets. Error bars are SD, *p≤0.05, ** =p < .01 by two tailed t-test for the difference between the proliferating subset average value and 1.0 (no difference between for Mϕ+C1q-LAL / Mϕ+LAL).

In the previous report from this lab, it was demonstrated that IL-27 and other regulatory cytokines were expressed at a higher level in C1q-polarized Mϕ relative to Mϕ ingesting LAL (Benoit et al., 2012). To validate that under the conditions used here for the MLR (X-VIVO-15 vs. HL-1 media) that IL-27 was increased, IL-27mRNA was assessed 4 – 7 hr after ingestion of apoptotic cells. In three different donors IL-27 expression was increased 3 – 6 fold in C1q-polarized Mϕ (Figure 3.7A) relative to Mϕ ingesting LAL. Taken together, these data suggest that soluble factors released by Mϕ that ingested C1q coated LAL contribute to the suppression of Th17 and Th1 cells.

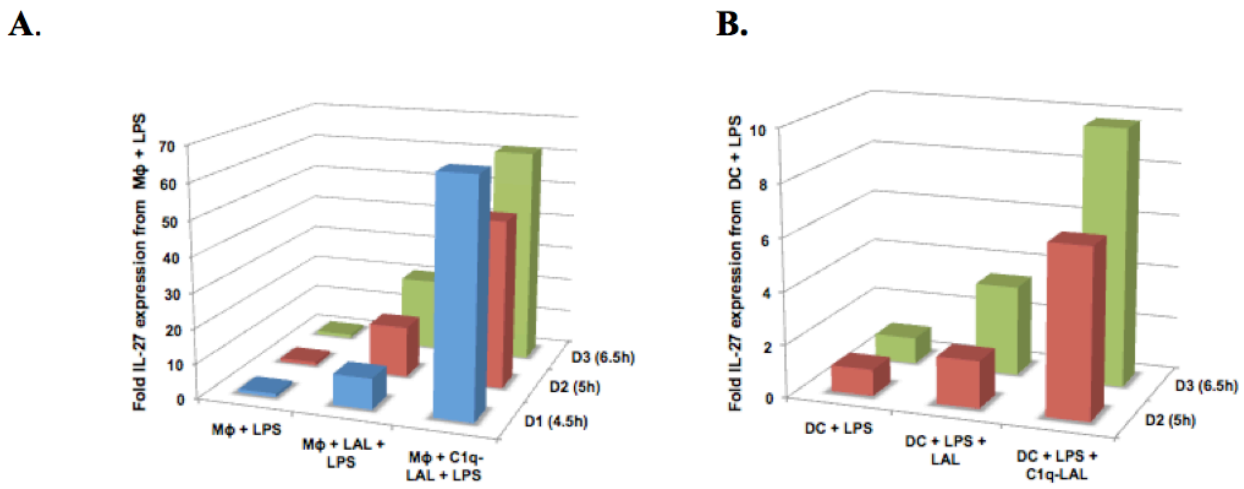


Figure 3.7 – Ingestion of apoptotic cells to which C1q is bound increases IL-27 mRNA in both Mϕ and DC. Mϕ (A) or DC (B) were incubated with LAL which were preincubated or not with C1q, at a 5:1 ratio for 1 h and after washing away uningested cells, were stimulated with 10 ng/ml LPS for 4.5 – 6.5 hours for 3 donors (D1, D2, D3). IL-27 mRNA levels were determined by qRT-PCR, and normalized to GAPDH mRNA. The data are presented as the fold increase from either Mϕ (A) or DC (B) stimulated with LPS alone.

C1q-polarized DCs suppress proliferation of autologous inflammatory T cells proliferation.

To elucidate whether C1q-polarized DC functionally modulate the adaptive immune responses, human autologous T cells were incubated with DC that had ingested LAL only or DC

that had ingested C1q-bound LAL (C1q-polarized DC) and the proliferation of CD4+ and CD8+ T cell subsets was assessed. Relative to DC ingesting LAL in the absence of C1q, C1q-polarized DC decrease autologous Th17 proliferation by an average of 53% ($n = 5$, $p<0.01$) (Figure 3.8 A – C, G, H) and Th1 proliferation by an average of 35% ($n = 5$, $p<0.01$) (Figure 3.8 D – F, G, H). As with the C1q-polarized macrophages, DC that had ingested C1q-LAL expressed higher levels of IL-27 mRNA (2.9 – 3.6-fold), as determined by qPCR (Figure 3.7B). Similar to the MLR experiments done with Mϕ and fresh T cells, DC ingesting C1q-LAL suppress freshly isolated Th17 proliferation by an average of 58% and Th1 to a lesser extent (10%) relative to DC ingesting LAL in two donors. Again, C1q-polarized DC did not have a consistent effect on the proliferation of either CD8+IFN-γ+ T cell proliferation or CD4+Foxp3+ or CD8+Foxp3+ subsets (data not shown).

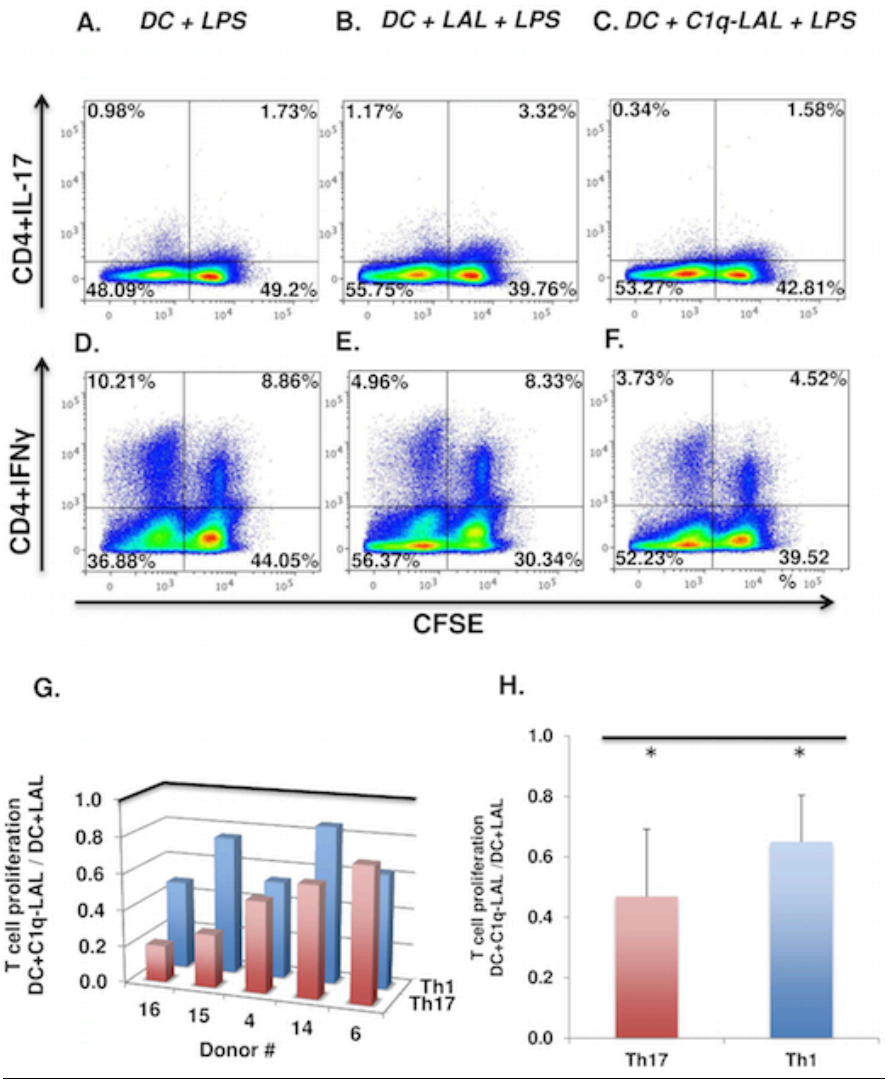


Figure 3.8 – C1q-polarized DC suppress autologous T cell proliferation of inflammatory T cell subsets. CFSE-stained autologous primary human T cells (95% CD3+, maintained in IL-7 for one week) were added (3:1) 24 hours after LPS treatment of DC that had ingested LAL (middle) or C1q-bound LAL (right). After 5 days, the non-adherent cells were removed, restimulated, processed and assessed as in Figure 3.3. Using Flow-Logic, cells were gated on α-CD4-PE and IL-17-PE-Cy7 (A – C) or and IFN-γ-APC-Cy7 (D – F). Data are representative of 5 independent experiments. Percent of total events is indicated in each quadrant. (G) The ratio of DC+C1q-LAL / DC+LAL for the percent proliferating Th17 and Th1 cells for each of 5 donors. Darkened line at 1.0 demarks no difference between for Mϕ+C1q-LAL / Mϕ+LAL) for the percent proliferating subsets. (H) Average value of 5 donors for fold change DC+C1q-LAL / DC+LAL for the percent proliferating subsets. Error bars are SD, *p<0.01 by two tailed t-test for the difference between the proliferating subset average value and 1.0 (no difference between for DC+C1q-LAL / DC+LAL).

Discussion

In this study, human Mϕ and DC ingesting late apoptotic cells to which C1q was bound suppressed the proliferation of both allogeneic and autologous inflammatory Th17 and Th1 T

cell subsets known to be elevated in autoimmunity (Alunno et al., 2012;Shin et al., 2011;Lazarevic et al., 2011). C1q attenuation of autologous Mϕ-mediated Th17 and Th1 cell proliferation was similar whether TLR4 ligand LPS or TLR7/8 ligand R848 was added prior to the MLR (Figure 3.4). This is notable given that TLRs 7 and 8 appear to play a significant role in autoimmunity (Krieg and Vollmer, 2007) and suggests that C1q is broadly suppressive of the Mϕ-mediated proliferation of these subsets. A persistent reduction in these populations over time during continuous autoantigen clearance could have biological consequences particularly in individuals in which there is a propensity for deficient or inefficient clearance or excessive tissue damage that exceeds the capacity for "silent" clearance of dying cells and debris. Additionally, C1q-polarized Mϕ had elevated surface expression of PD-L1 and PD-L2 and significantly suppressed induction of the surface co-stimulatory receptor CD40 relative to Mϕ ingesting LAL alone. Furthermore, C1q-polarized DC exhibited elevated surface expression of PD-L2 and CD39 and significantly suppressed induction of the surface co-stimulatory receptor CD86. These observations establish a functional consequence of the C1q-induced changes in APC on elements of the adaptive immune response in *ex vivo* conditions, extending previous work demonstrating that primary human Mϕ and DC ingesting C1q-bound LAL upregulate and secrete significantly more anti-inflammatory cytokines such as IL-27 and IL-10 (Benoit et al., 2012;Fraser et al., 2009), produce significantly less pro-inflammatory cytokines such as IL-1β, TNF-α and IL-12 (Fraser et al., 2007;Fraser et al., 2009;Fraser et al., 2006;Nauta et al., 2004) and in the case of Mϕ have reduced inflammasome activation relative to Mϕ ingesting LAL alone (Benoit et al., 2012) (Figure 3.9).

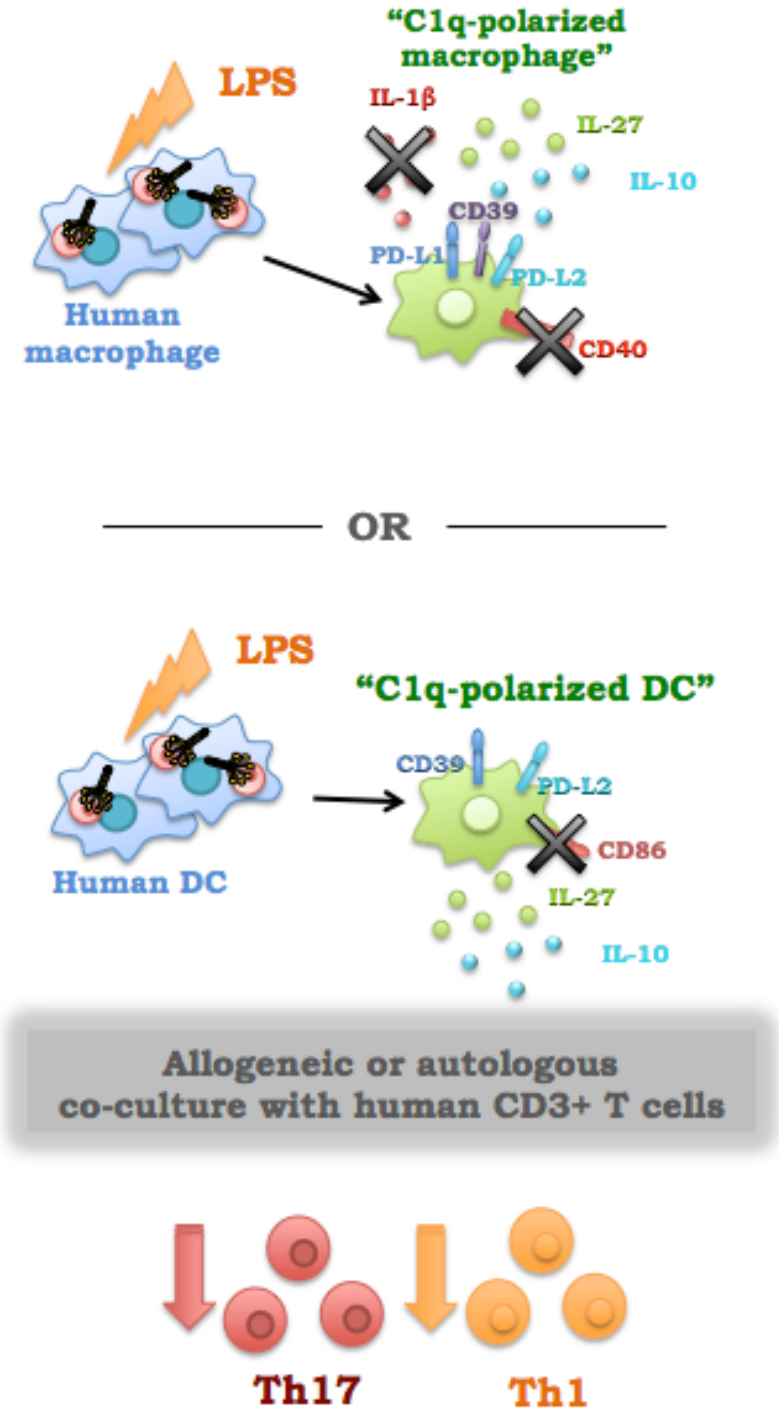


Figure 3.9 – Summary of complement protein C1q effects on human macrophage and dendritic cell polarization and effector T cell proliferation. Human monocyte derived macrophages or DCs ingest C1q-bound apoptotic lymphocytes after which they were stimulated with low level of TLR agonist. The "C1q polarized macrophages" exhibit elevated PD-L1 and PD-L2 and reduced CD40 surface expression and produce greater IL-27 and IL-10 and less IL-1β than macrophages that had ingested LAL alone. "C1q polarized DCs" exhibit elevated PD-L2 and CD39 and reduced CD86 surface expression and produce more IL-10 than DCs that had ingested LAL alone. The proliferation of allogeneic and autologous Th17 and Th1 T cell subsets was significantly diminished in an MLR when cultured with C1q-polarized APC vs. APC ingesting LAL without C1q.

PD-L1 and PD-L2 bind the T cell inhibitory receptor PD-1 to reduce effector T cell activation (Fife et al., 2009), and CD39 decreases inflammasome activation (by reducing extracellular ATP as a secondary signal for the inflammasome) and limits Th17 and Th1 generation (Mascanfroni et al., 2013). Given that IL-27 is known to inhibit the Th17 subset (Mascanfroni et al., 2013;Batten et al., 2006;Diveu et al., 2009;Stumhofer and Hunter, 2008), possibly through up-regulation of PD-L1 (Hirahara et al., 2012) and/or CD39 (Mascanfroni et al., 2013), the increased IL-27 observed in the case of C1q-polarized Mϕ (Figure 3.6A) may contribute to the reduced Th17 proliferation in part via regulation of these cell surface markers. The C1q-mediated suppression of CD40 induction relative to Mϕ that have ingested LAL without C1q may be attributed to the decrease in TNF-α and IL-1β previously observed in C1q-polarized Mϕ (Fraser et al., 2009;Benoit et al., 2012). CD40 is known to interact with CD40L on T cells to enhance Th1 responses (Baruah et al., 2009;Chatzigeorgiou et al., 2009), and thus suppression of CD40 may partially contribute to the decreased stimulation of T effector subsets by C1q-polarized Mϕ. Benoit et al (Benoit et al., 2012) also found a C1q-mediated enhancement of expression of IL-13, a typical M2-driven cytokine, IL-33, a member of the IL-1 family that can amplify M2 (alternative) polarization of macrophages, IL-37 (IL1F7), a natural suppressor of innate inflammatory responses (Nold et al., 2010). Since C1q-LAL clearly enhances IL-27 expression in both HL-1 and X-VIVO-15 media (Figure 3.7 A, B), it is likely that in this media, C1q-LAL promotes the expression of these other negative modulators as well, and is consistent with the negative regulatory effect of the supernatants from C1q-polarized Mϕ on the Th17 and Th1 subsets.

Previously, we have shown that C1q suppresses DC production of IL-1β, TNF-α and IL-

12 and enhances DC production of IL-10 (Fraser et al., 2009). This C1q-induced cytokine milieu may contribute to the effect of C1q-polarized DC on diminished Th17 and Th1 proliferation given that IL-12 induces Th1 cells (Hsieh et al., 1993) and IL-1β is required for Th17 cell induction (Ghoreschi et al., 2011). The enhanced C1q-mediated PD-L2 expression and/or suppressed CD86 (Caux et al., 1994) expression and/or enhanced IL-27 expression (Figure 3.7B) may also be contributing to the suppression of Th1 and Th17 subsets (Latchman et al., 2001).

The inhibition of Th1 proliferation by C1q-polarized Mϕ and DC is consistent with the upregulation of IL-10 by C1q-polarized Mϕ and DC (Fraser et al., 2009;Benoit et al., 2012) and the known ability of IL-10 to suppress the Th1 subset in certain contexts (Villegas-Mendez et al., 2013;O'Shea and Paul, 2010;Ito et al., 1999). Indeed, our data demonstrate that conditioned media from C1q-polarized Mϕ added to untreated M-CSF-derived Mϕ resulted in a reduction in Th17 and Th1 proliferation at MLR day 6 relative to that seen with conditioned media from Mϕ that ingested LAL without C1q, similar to, though smaller in magnitude to the effect of the C1q-polarized Mϕ themselves (Figure 3.6). Elevated mRNA levels for IL-27 were seen in both C1q-polarized Mϕ and DC (Figure 3.7A, B), consistent with the hypothesis that these soluble factors and others are critically contributing to the suppression of Th17 and Th1 lineage proliferation. Thus the effect of C1q may be to modulate Mϕ and DC responses to low levels of DAMPs accumulating during cell death/tissue repair (Bell et al., 2006) to suppress expansion of potentially damaging Th17 and Th1 cells in the tissue in the absence of significant infection.

The allogeneic MLR employed here may be recapitulating some of the conditions occurring during allograft transplantation and in this way C1q may play an anti-inflammatory role in suppressing T cell-mediated allograft rejection, consistent with previous *in vivo* mouse data (Baruah et al., 2010). However, co-culture of C1q-polarized Mϕ and DC with autologous T

cells is a situation that more closely mimics the *in vivo* clearance of apoptotic cells. The T cells that were maintained in IL-7 were nearly 100% CD45RA+, and thus while they may not be strictly naïve (Sathaliyawala et al., 2013), freshly isolated autologous T cells responded similarly. Given that C1q-polarized Mϕ and DC consistently suppressed Th17 and Th1 proliferation even in the absence of substantial Foxp3+ T cells, it is likely that the APC themselves (via cell interaction and/or their secreted products) are contributing substantially to suppression of the Th17 and Th1 subsets rather than an indirect C1q-mediated decrease in these lineages by way of CD4+Foxp3+ or CD8+Foxp3+ mediated suppression.

It remains to be determined what molecular mechanisms are involved in the synergistic effect of C1q-LAL and TLR agonists. Our earlier studies showed that some C1q-induced signaling events are independent of TLR ligation such as the induction of NFkB p50p50, a negative regulator of proinflammatory activation which is induced both in the presence and absence of LPS (Fraser et al., 2007). Thus, C1q induced p50p50 complexes may "compete" for TLR-triggered proinflammatory NFkB complexes (p65p65 or p65p50) thereby limiting proinflammatory cytokine production. As a result, the suppressive effects are seen only in the presence of a TLR agonist, which would otherwise induce proinflammatory cytokines. In addition, C1q-LAL may alter "cargo" trafficking, processing and/or antigen presentation which would then have downstream effects on T cell subset activation / proliferation. Such alterations have recently been reported for C3 in the murine system although the end effect of this was enhanced antigen presentation, as may be expected, to facilitate an immune response to a complement activating invader (Baudino et al., 2014).

While at first surprising that addition of autologous LAL to the Mϕ or DC increased overall levels of proliferation as compared to the Mϕ or DC + LPS in the absence of LAL, APC

ingestion of apoptotic cells has previously been shown to promote T cell proliferation in a primary human autologous mixed leukocyte reaction (Amel Kashipaz et al., 2002;Chernysheva et al., 2002). Since Mϕ and DC are maintained in RPMI media + 10% FBS (containing alloantigens) and are likely sampling that environment during their 7 day differentiation from freshly isolated peripheral blood monocytes, it has been suggested that they are presenting these alloantigens to the T cells, which then proliferate in response (Mallone et al., 2011).

Intriguingly, the C1q receptor responsible for these effects remains to be identified. C1q has been shown to interact with several different myeloid cell surface molecules, including LAIR-1, resulting in suppression of human monocyte to dendritic cell differentiation and activation (Son et al., 2012). Additionally, SCARF1 (Ramirez-Ortiz et al., 2013) and calreticulin (Byrne et al., 2013) have also been implicated in the C1q-mediated enhancement of clearance of apoptotic cells. We found that SCARF1 is expressed on the primary human Mϕ and DC used in our experiments; however LAIR-1, while detected on freshly isolated monocytes, is not expressed on the primary human Mϕ or DC (data not shown); thus future experiments should be able to determine the C1q receptor(s) responsible for the effect of C1q-bound apoptotic cells on Mϕ and DC cytokine production, surface molecule expression and Mϕ and DC-mediated T cell activation that we report here.

This study adds to a growing body of literature demonstrating a role for complement proteins in modulating T cell function during disease and under homeostatic conditions (reviewed in (Kemper and Kohl, 2013)). Indeed, it is becoming increasingly clear that complement proteins, traditionally thought to regulate only innate immune cells, are also capable of influencing the adaptive immune system. However, to our knowledge, this is the first instance demonstrating the immunoregulatory role of C1q on Mϕ- and DC-mediated T cell activation in

an entirely human system with the more physiologic stimulus of autologous dying cells. Only a couple of studies to date have investigated whether C1q plays a role in modulating T cell activation. Cutler et al (Cutler et al., 1998) demonstrated that in C1q knockout mice, antigen-specific T cells exhibited a significant reduction in IFN-γ production compared to control mice. Similarly, a more recent study from the same group (Baruah et al., 2009) used DCs from C1q knockout mice and found that in this context, exogenously added C1q augments the production of IL-12 by DCs and increases the number of CD4+IFN-γ+ (Th1) and CD8+IFN-γ + T cells in response to DC CD40 ligation although no difference was seen in response to TLR ligands. However, these experiments were conducted in mice, with complete genetic knockout (lifetime) of C1q. Indeed, though routinely used in studies of inflammation, the mouse model fails to recapitulate some human inflammatory diseases (Seok et al., 2013;Osterburg et al., 2013;Mestas and Hughes, 2004). Importantly, Teh and colleagues used C1q-treated primary human DCs and found that C1q decreased IL-12 and IL-23 production as well as reduced Th1 and Th17 induction from allogeneic CD4+ T cells in a mixed leukocyte reaction (MLR) (Teh et al., 2011). These data are consistent with the effect of C1q bound to apoptotic cells that we report here and suggest that C1q influences human DCs to skew T cells towards a more anti-inflammatory function, consistent with a role of C1q in promoting tolerance while avoiding autoimmunity (Skapenko et al., 2005;Sathaliyawala et al., 2013).

However, unique to the studies presented here, human Mϕ- and DC-mediated CD4 and CD8 T cell responses were examined specifically as the functional consequence of the ingestion of C1q-coated human autologous apoptotic cells (as opposed to C1q immobilized on a plate (Teh et al., 2011) or using transformed cell lines as the source of apoptotic cells). Finally, our novel, entirely primary human cell system provides a better approximation of the effects of C1q on T

cells found in tissue (e.g., skin, liver, spleen, kidneys, etc.), where C1q can be secreted by myeloid cells in the absence of the enzymatic C1r and C1s subcomponents of C1 and thus in the absence of activation of the complement cascade (Fraser et al., 2009;Bensa et al., 1983) and thus would be present during the phagocytosis of apoptotic cells by these cells. As the tissue can become the site of inflammation in autoimmune scenarios, understanding what regulates Mϕ and DC responses in tissues that ultimately influence T cell activation is critical.

In summary, the data presented here identify the ability of C1q alone to control the Mϕ and DC mediated T cell immune response in primary human cells to apoptotic cells, a physiologically relevant self cargo. Importantly, while it has been long known that SLE is initiated / propagated in the absence of C1q or in the case of reduced C1q function (Martens et al., 2009) in humans, our studies provide a pathway by which C1q may critically contribute to the regulation of the adaptive immune response and prevention of lupus-like autoimmunity in normal individuals. The novel combination of human peripheral blood derived T cells, autologous antigen presenting cells and autologous apoptotic cells with or without the addition of purified human C1q is unique among studies approaching this topic, and avoids the caveats in previously published studies performed in mice, with transformed/foreign cell lines and with non physiologic presentation of the C1q molecule. These new findings demonstrate that C1q bound to dying cells influences Mϕ- and DC cytokine production, surface molecule expression and subsequent T cell subset proliferation, “sculpting” the adaptive immune system to avoid autoimmunity and promote tolerance. Importantly, these studies may lead to the identification of novel target pathways for therapeutic and/or preventative intervention in SLE and other autoimmune diseases.

Chapter 4 – Summary and Future Directions

Summary of Results

Systemic lupus erythematous (SLE) pathogenesis is characterized by lymphoproliferation, expansion of autoreactive T and B cells and nuclear autoantibody production, resulting in immune complex deposition and subsequent tissue damage (Tsokos, 2011). One mechanism implicated in the initiation and/or progression of SLE is a deficiency in apoptotic cell clearance as that would result in the lysis or leakage of nuclear and inflammatory contents as the uningested cells undergo secondary necrosis (Potter et al., 2003). This could lead to auto-antibodies to self proteins and aberrant myeloid and T cell activation. It has long been known that the complete genetic absence of complement protein C1q results in SLE-like autoimmunity with close to 100% penetrance (Botto and Walport, 2002). Thus, it is hypothesized that C1q may help avoid autoimmunity and prevent SLE in normal individuals. However, the molecular and cellular mechanisms for this striking functional effect of C1q are only beginning to be elucidated. There is strong motivation to uncover and delineate these pathways since knowledge of specific C1q-mediated events could identify physiologically relevant molecules involved in SLE pathogenesis and lead to targeted therapeutics for these individuals and potentially others with inflammatory autoimmune disorders involving elevated or unregulated adaptive immune responses.

SLE is thought to result from defects in apoptotic cell clearance. Work in our lab and others has demonstrated a role for C1q in enhancing apoptotic cell clearance. In this thesis, a novel autologous system using primary human leukocytes was applied to characterize the effect

of C1q on Mϕ and DC functional polarization after the uptake of apoptotic cells. C1q bound to autologous AL modulated Mϕ gene expression associated with JAK/STAT signaling, chemotaxis, immunoregulation and NLRP3 inflammasome activation in LPS-stimulated Mϕ. C1q also increased STAT1 phosphorylation and sequentially induced type I interferons (IFN-s), IL-27 and IL-10 in LPS-stimulated Mϕ. IL-27 was also increased in Mϕ when incubated with AL conditioned media (Figure 2.6), suggesting that AL are a likely a source of DAMPs associated with sterile inflammation as would occur during the initiation and propagation phases of SLE. Additionally, adding the collagen-like C1q tail domain to Mϕ cultures thought to be responsible for the C1q interaction with and functional effects on Mϕ) prevented the induction of type I IFN-s and IL-27 in a dose dependent manner, suggesting a multimeric interaction (and thus receptor clustering) is necessary for the signaling. Furthermore, antibody neutralization of type I IFN-s partly prevented IL-27 induction by C1q, implying that the C1q-mediated increase in IL-27 is due at least in part to the C1q enhancement of type I IFN-s. C1q bound to LAL also induced surface expression of PD-L1 and PD-L2 (known to inhibit antigen-dependent T cell activation) in Mϕ while decreasing CD40 expression relative to LAL alone. Finally, C1q-LAL decreased procaspase-1 cleavage and caspase-1 dependent cleavage of IL-1β, signifying a potent inhibitory effect of C1q on inflammasome activation. Hence, in this physiologically relevant system comprised of entirely autologous primary human cells, C1q powerfully polarizes primary human Mϕ towards an anti-inflammatory function.

In similar studies in which primary human immature DCs ingested autologous C1q-LAL or LAL, DCs ingesting C1q-LAL exhibited increased IL-27 transcript levels. C1q-LAL also triggered enhanced PD-L2 and CD39 (an ectoenzyme responsible for inhibiting the inflammasome) expression and suppressed CD86 expression. These data corroborate and extend

earlier results showing that C1q suppresses DC inflammatory cytokine while enhancing anti-inflammatory cytokine production (Teh et al., 2011;Fraser et al., 2009;Santer et al., 2012) and that C1q inhibits DC CD86 expression (Castellano et al., 2007). These results demonstrate that C1q polarizes primary human DCs towards an immunoregulatory functional phenotype.

Thus, while it is becoming somewhat clearer how C1q affects primary human myeloid cell function when presented via the more physiologic stimulus of an autologous apoptotic cell, there have been no prior studies investigating the influence of C1q on myeloid cell-mediated T cell activation in this context. Given the role that the effector T cells Th17 and Th1 play in SLE-related inflammation and tissue damage and autoantibody production (Shin et al., 2011;Lazarevic et al., 2011;Liu and Davidson, 2012;Ding et al., 2013;Pisitkun et al., 2012;Miyake et al., 2011;Wen et al., 2013), a major aim of my thesis has been to characterize the effect of C1q on Mϕ and DC-mediated Th17 and Th1 cell subset proliferation. Extending the system of primary human cells described above, I added primary human autologous or allogeneic T cells (CD3+) purified from peripheral blood. In this system, I discovered that both Mϕ and DC ingesting autologous C1q-bound LAL suppressed the induction of Th17 and Th1 cell proliferation relative to Mϕ and DC ingesting C1q without LAL. In addition, a trend toward C1q-LAL mediated allogeneic Treg enhancement was observed. Given the crucial role of Th17 and Th1 subsets in promoting autoimmunity, these data identify a novel pathway by which C1q interaction with myeloid cells may prevent the initiation and propagation phases of autoimmunity and promote tolerance. Thus, these results could lead to the development of potential therapeutic targets to prevent and control autoimmunity and inflammation.

Future Directions

To more fully understand the link between the initial C1q-triggered events, future studies should focus on more fully elucidating (a) the C1q-LAL mediated signaling pathways in Mϕ and DC and (b) the effect of C1q on Mϕ and DC phagosome maturation and antigen processing that leads to the observed Mϕ and DC functional effects including cytokine, surface marker and eventual APC-mediated T cell modulation. In addition, the C1q receptor(s) responsible for the observed C1q-triggered functional effects remain to be identified. Given the observed anti-inflammatory events triggered by C1q, knowledge of the C1q receptor(s) involved in these effects could pinpoint clear therapeutic target(s) for amplifying the C1q-mediated pathways in autoimmune / inflammatory diseases such as SLE or inhibiting these pathways in the case of cancer.

Identifying C1q-induced signaling pathways

In order to more fully understand the mechanism of how C1q is triggering the observed functional effects on Mϕ and DC cytokine production, surface marker expression and APC-mediated T cell subset modulation, a key goal for future studies should be to characterize the signaling events triggered by C1q-LAL and C1q-EAL interaction with primary human Mϕ and DC during the phagocytosis of these LAL. As noted here, C1q bound to LAL modulates the expression of genes associated with JAK/STAT signaling (Clarke et al., 2011) and NLRP3 inflammasome activation in LPS-stimulated Mϕ (Benoit et al., 2012). Western blot and flow cytometry analysis demonstrate that C1q-bound late apoptotic lymphocytes induces rapid (30

min) STAT1 phosphorylation at Tyr701 after LPS addition (Figure 2.4). Microarray data indicate that LAL coated with C1q increase Mϕ IFN-AR2, which may be required for IFN-α–mediated IL-27 secretion (Piganis et al., 2011). The C1q-mediated increase in Mϕ STAT1 phosphorylation and IFN-α production coincide with a decrease in inflammasome activity (Clarke et al., 2011), which is in agreement with a recent report demonstrating STAT1- and IFN-α-mediated inhibition of the inflammasome (IFN-α is produced via activation of STAT1) (Guarda et al., 2011). Likely in response to these early events, it was found that C1q sequentially induces IFN-α, IL-27 and IL-10 in LPS-stimulated Mϕ when incubated with apoptotic lymphocyte conditioned media. Moreover, a C1q-mediated increase in PD-L1 is also observed (Figure 3.1), as well as a suppression of co-cultured Th17 cells, likely in response to the enhanced IL-27 expression (Figure 3.7) (Karakhanova et al., 2011). However, (aside from STAT1), additional signaling molecules involved in these C1q-mediated effects remain to be identified. Given that in the presence of low levels of LPS C1q-LAL is triggering Mϕ production IFN-α, IL-27 and IL-10 in sequential fashion (above that induced by LAL + LPS or LAL alone) (Karakhanova et al., 2011), just as Iyer et. al. found in the case of LPS-stimulated murine Mϕ (Iyer et al., 2010), it is likely that C1q is accelerating the pathways downstream of TLR ligation in order to resolve inflammation. In murine LPS-stimulated Mϕ, it was also found that IFN--α is produced in response to STAT1 phosphorylation, and IL-27 and IL-10 are synthesized in response to both STAT1 and STAT3 phosphorylation (Iyer et al., 2010). Data from LPS-treated murine Mϕ showed that IFN--α and IL-27 exert differential effects on the IL-10 promoter, but that together the cytokines share a common regulatory region located between −78/−118 relative the transcription start site that corresponds to an Sp1 binding site (Iyer et al., 2010;Mosser and Zhang, 2008). It is also known that phosphorylation of JAK1, Tyk2 and JAK3 is involved in the

activation of STAT1 and STAT3 (Murray, 2007). It will be relevant to evaluate whether the C1q-LAL mediated enhancement of sequential IFN-α, IL-27 and IL-10 production is occurring via phosphorylation of these JAKs and STATs and / or different transcription factors in both Mϕ (which exhibit enhanced IFN-α, IL-27 and IL-10) and DC (which have elevated IL-27 and IL-10).

In primary human Mϕ, C1q-LAL trigger other signaling pathways leading to the reduction of pro-inflammatory cytokines such as TNF-α (Benoit et al., 2012;Fraser et al., 2009). Interestingly, microarray data from Mϕ show that C1q-bound EAL and LAL increase PPARγ coactivator 1-α (PGC-1α) levels, suggesting that C1q may increase PPARγ signaling (Benoit et al., 2012). We also observe an induction of AMPK (catalytic α subunit)—which is downstream of PPARγ (Hardie, 2007)—in Mϕ ingesting both C1q-EAL and C1q-LAL by microarray. Data from qPCR confirmed this result, demonstrating that Mϕ ingesting C1q-bound EAL have significantly increased levels of AMPK mRNA. Interestingly, it was recently reported that increased AMPK activity results in AMPK attenuates LPS and CD40-mediated proinflammatory activity of myeloid cells (Carroll et al., 2013). Since C1q-LAL decrease Mϕ ingesting C1q-bound apoptotic cells exhibit decreased CD40 expression relative to Mϕ ingesting apoptotic cells alone (Figure 3.1) and preliminary qPCR (Figure 4.1) and microarray data (Benoit et al., 2012) demonstrate that Mϕ interaction with C1q on apoptotic cells also increases AMPK expression, it is possible that Mϕ ingesting C1q-bound apoptotic cells exhibit increased AMPK activity leading to the observed decrease in CD40 surface expression.

Additionally, phosphorylated STAT6 is known to interact with PPARγ and facilitate its binding to DNA (Szanto et al., 2010). It has also been shown STAT6 activation leads to the upregulation of PD-L2 on Mϕ and DC (which is upregulated on Mϕ and DC by C1q in our

system, Figure 3.1 and Figure 3.2) and the suppression of T cell activation (Huber et al., 2010). Therefore it is plausible that Mϕ and DC interacting with C1q-coated apoptotic cells may be inducing PPARγ translocation and response element binding in cooperation with STAT6. Since STAT6 activation leads to AMPK phosphorylation (Sag et al., 2008)*,* it's possible that C1q may trigger STAT6 and AMPK activation, ultimately to the observed increased PD-L2 expression.

Intriguingly, C1q has been shown to upregulate the expression of Mer tyrosine kinase (MerTK) (Galvan et al., 2012b), a macrophage receptor for apoptotic cells. Recently, it was demonstrated that murine Mϕ interacting with immobilized C1q triggered AMPK activation, and silencing of AMPK expression inhibited C1q-dependent phagocytosis (Galvan et al., 2014). Thus, it is reasonable to postulate that primary human Mϕ interacting with C1q-bound apoptotic cells may stimulate similar AMPK activation.

Furthermore, a recent report showed that AMPK negatively regulates LPS-induced IκB-α (negative regulator of NFκB signaling) degradation in Mϕ (Sag et al., 2008). Thus it is possible that C1q-bound apoptotic cells may be reducing pro-inflammatory molecules in Mϕ and DC (Fraser et al., 2009;Benoit et al., 2012) by either stabilizing IκB-α or by increasing NFκB p50p50 dimers as we have observed in monocytes treated with immobilized C1q (Fraser et al., 2007), which compete with p50p65 heterodimers for binding to NFκB response elements but do not activate transcription. TLR-ligation of the NFkB pathway leads to an increase in pro-inflammatory cytokines, including IL-1β, IL-18, IL-12, etc. (Dunne et al., 2010) but Mϕ ingesting C1q-coated apoptotic cells decreases these cytokines (Fraser et al., 2009;Benoit et al., 2012) and DC interacting with C1q on apoptotic cells decreased CCL3 and increased IL-10 (Fraser et al., 2009) and IL-27 (Figure 3.7). The signaling mechanism(s) responsible for this C1q effect on decreasing pro-inflammatory cytokines in Mϕ and DC and surface markers such as

CD40 in Mϕ (Figure 3.1) or CD86 in DC (Figure 3.2) ingesting autologous apoptotic cells to which C1q is bound remains to be identified.

Currently, there is no reported cooperative or synergistic intersection between the STAT1 / STAT3 pathway that leads to sequential upregulation of Type I IFN-s, IL-27 and IL-10 (Iyer et al., 2010) and the AMPK, PPARγ or NFkB (though NF-kB–driven SOCS3 expression negatively regulates STAT3 pathways in cultured primary human brain cancer cells (McFarland et al., 2013)described above in either human or murine Mϕ, DC or any other leukocyte. Thus, the C1q effect on each of these pathways is likely distinct, and as such, C1q is probably triggering several different pathways, which when summed result in the observed effects on cytokines, surface molecules and APC-mediated T cell activation. A hypothetical schematic outlining these potentially C1q-modulated pathways is given in Figure 4.1 Future studies could use an siRNA knockdown approach suitable for primary human Mϕ and DC (Troegeler et al., 2014) to evaluate the role of the relevant signaling molecules on the observed C1q-mediated effects on Mϕ and DC cytokine modulation and Th17 and Th1 cell subset inhibition during Mϕ and DC clearance of apoptotic cells.

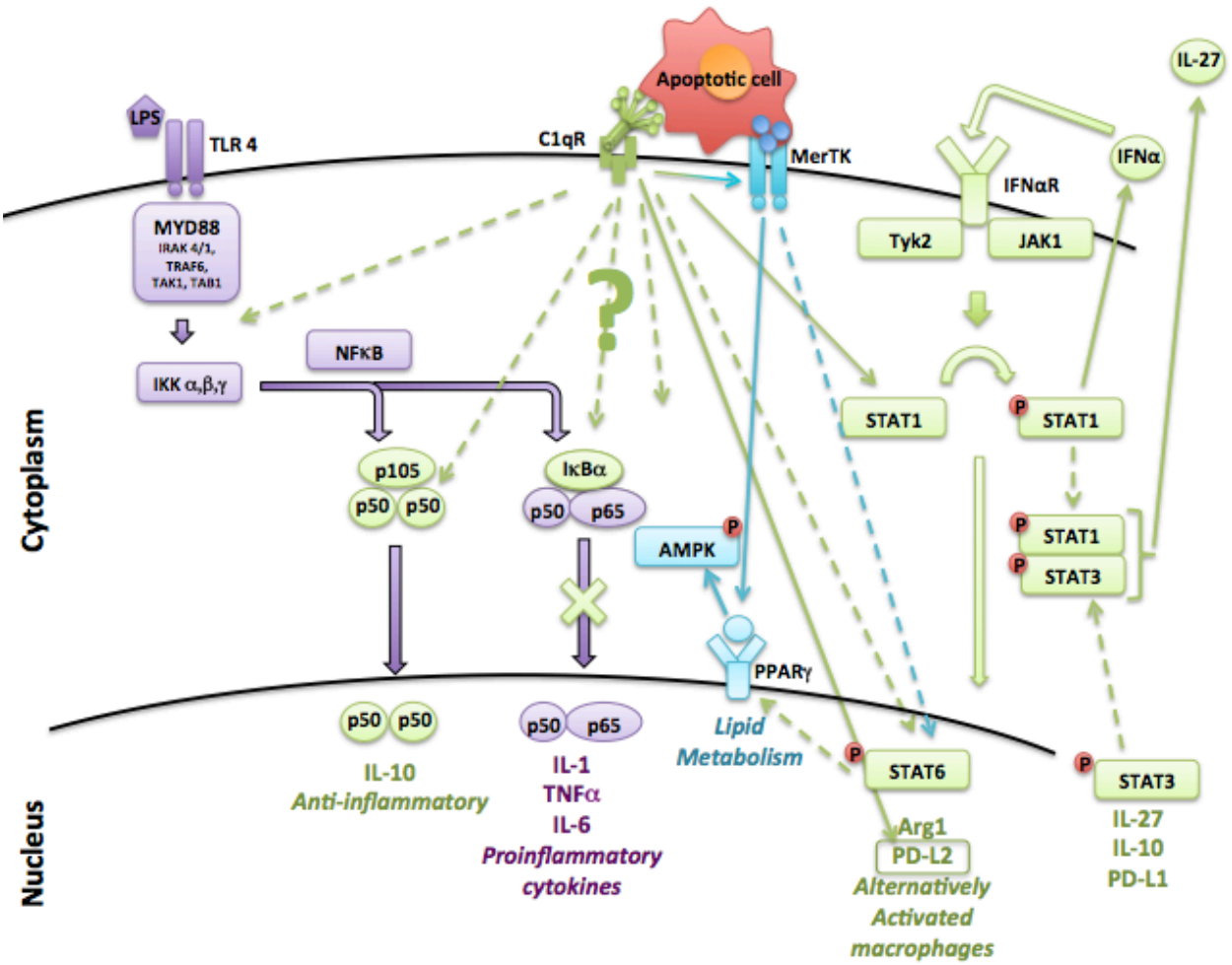


Figure 4.1 – Hypothetical schematic of pathways potentially modulated by C1q. Purple shading designates TLR-mediated signaling (3-6) which we trigger here with 10 ng/ml LPS stimulation. Green shading represents candidate (dotted lines) or determined (solid line) C1q signaling pathways (Fraser et al., 2007;Fraser et al., 2009;Benoit et al., 2012;Clarke et al., 2011). Blue represents signaling via macrophage receptor(s) that contribute to apoptotic cell recognition (e.g., MerTK) (Galvan et al., 2012a;Benoit et al., 2012;Majai et al., 2010;Mukundan et al., 2009;Roszer et al., 2011;Zizzo et al., 2012;Byrne et al., 2013).

Evaluating the effect of C1q on phagosome maturation

In addition to directly initiating early signaling events (possibly in response to receptor ligation), C1q may be altering phagosome maturation and intracellular trafficking of ingested particles / antigens which would lead to C1q-modulation of Mϕ and DC polarization and subsequent T cell activation. Indeed, the C1q-mediated consequences on the maturation of the apoptotic cell vacuole are not well characterized. Preliminary data collected in the Tenner lab in pursuit of this effort indicate that C1q may enhance the uptake of apoptotic cells by promoting

Mϕ macropinocytosis. Confocal microscopy data indicate that significantly more FITC-Dextran (a soluble fluorescent molecule that can be ingested only by macropinocytosis) co-localizes with C1q-bound apoptotic cells ingested by Mϕ than with ingested apoptotic cells not bound to C1q.

Additionally, and particularly relevant to the suppressive effect of C1q on Th17 and Th1 cell activation, preliminary data indicate that C1q accelerates the maturation of the apoptotic cell vacuole. Specifically, C1q-bound apoptotic cell vacuoles acquire the late endosomal marker LAMP-1 more rapidly than vacuoles with apoptotic cells not bound to C1q. Faster vacuole maturation may result in shorter antigen presentation, whereas delayed maturation may result in more sustained antigen presentation (Baudino et al., 2014), suggesting that C1q may reduce the time of presentation of self antigens derived from ingested apoptotic cells and in this way attenuate self-reactive T cell activation.

In addition, preliminary data indicate that C1q-LAL increase the activation of Akt, which is responsible for class I PI3K activation (Thi and Reiner, 2012). While no quantitative difference was detected by western blot, C1q-LAL enhanced the accumulation of pAkt around the apoptotic cell vacuole, suggesting that C1q increases PI3K activation at vacuole site rather than in the whole cell to promote maturation toward phagolysosome, which is required at multiple steps of particle uptake and subsequent vacuole trafficking (Kinchen and Ravichandran, 2008).

Taken together, these preliminary results suggest that C1q directly modulates early steps in the phagocytosis process in macrophages, regulating the activation of the PI3K-Akt pathway and the subsequent maturation of the apoptotic cell vacuole. Further investigation using specific PI3K inhibitors will be needed to confirm whether these C1q-triggered pathways are involved in

the subsequent modulation of inflammation and Th17 and Th1 cell subset inhibition during the Mϕ clearance of apoptotic cells.

Identifying the Mϕ and DC C1q receptor(s) involved in apoptotic cell clearance

Another fascinating and highly relevant question is the identification of the C1q receptor(s) involved in apoptotic cell clearance, as these receptor(s) would be responsible for the C1q effects observed in the experimental system reported here and thus are likely involved in preventing SLE and other autoimmune / inflammatory diseases. To date, numerous C1q receptors that interact with the collagen-like domain of the molecule and are potentially involved in apoptotic cell clearance have been identified, including but not limited to calreticulin (Paidassi et al., 2011;Duus et al., 2010;Ogden et al., 2001), DC-SIGN (Hosszu et al., 2012), RAGE (Ma et al., 2012b), and SCARF1 (Ramirez-Ortiz et al., 2013) although these results are still not replicated and remain controversial. Of note, while the authors claimed murine SCARF1 binds C1q *in vitro*, direct binding was not demonstrated; C1q binding to SCARF1 was measured in an indirect reporter assay. SCARF1 was also shown to mediate the CD8α+ DC and Mϕ interaction with, and ingestion of, apoptotic cells *in vitro* and *in vivo* and avoid autoimmunity *in vivo* (Ramirez-Ortiz et al., 2013). Given that mice deficient in SCARF1 develop autoimmunity, similar to $C1q^{-/-}$ mice on certain strain backgrounds (Botto et al., 1998;Mitchell et al., 2002;Miura-Shimura et al., 2002), the possibility that SCARF1 may be a critical receptor for apoptotic cells in humans is an intriguing one. I have collected preliminary data demonstrating that SCARF1 is upregulated by both Mϕ and DC ingesting C1q-LAL relative to Mϕ and DC ingesting LAL (Figure 4.2).

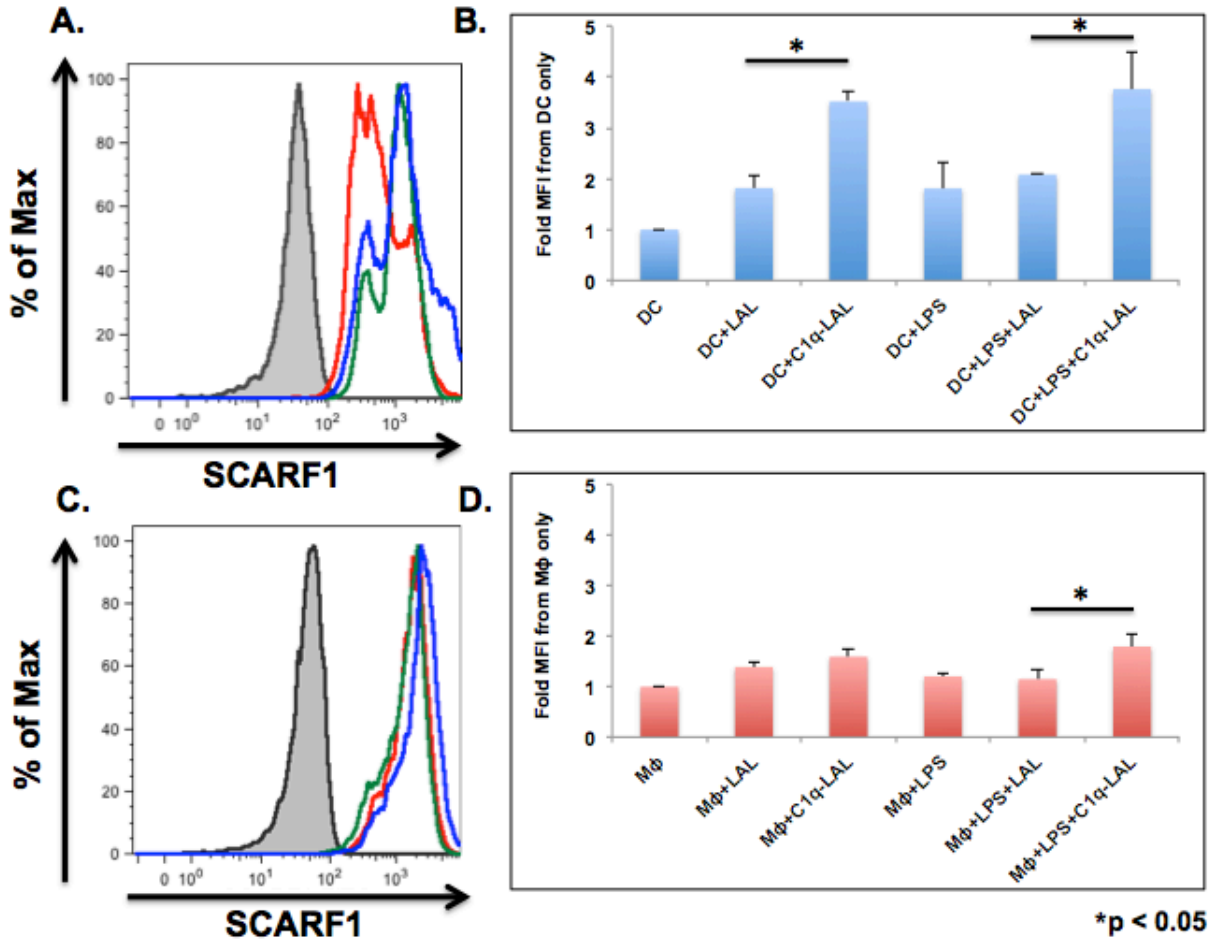


Figure 4.2: C1q increases SCARF1 expression in DC and Mϕ. C1q-coated autologous apoptotic lymphocytes (C1q-LAL) (blue) or LAL only (green) were added to GM-CSF + IL-4 monocyte-derived dendritic cells (DC) (A, B) or M-CSF-monocyte derived Mϕ (C, D) in a 5:1 ratio for 1h, after which the uningested apoptotic cells were washed away. DC and Mϕ were then stimulated or not with 10 ng/ml LPS in X-VIVO-15 for 24h. The adherent cells were harvested, stained with SCARF1-FITC and read by flow cytometry. Controls were DC without LAL (red). Grey shading (A, C) indicates PE-isotype control. Representative FACS are shown for samples with LPS added (A, C). Average fold change (fold) MFI relative to DC (B) or Mϕ only (D) for 2 independent experiments. *p< 0.05 by Students T-test. Error bars are S.D.

These results suggest the possibility that C1q could be upregulating its own receptor. Future studies should explore whether SCARF1 is necessary and sufficient for C1q-mediated Mϕ and DC uptake of apoptotic cells and the functional effects on APC production of cytokines, expression of surface molecules and APC-mediated T cell activation that is reported here.

Concluding Statement

Taken on the whole, the data presented in this thesis identify a novel function for complement protein C1q as an immunological rheostat and potent suppressor of innate and adaptive immune activation and inflammation in the physiological context of dying cells; a sterile inflammatory situation that could trigger autoimmunity. The pathways identified in these results pinpoint targets for preventative and therapeutic intervention in SLE and potentially other autoimmune / inflammatory diseases. In addition, given that these data were collected in an entirely primary human cell system largely under autologous conditions, the results form the basis for a more physiologically relevant understanding of what may be occurring in the initiation and propagation phases of SLE. Future studies should continue to explore the molecular mechanisms observed in the C1q-mediated Mϕ and DC cytokine effects and subsequent Mϕ and DC-mediated T cell proliferation. In particular, it will be interesting and useful to identify early C1q-initiated signaling events and continue to explore the effect of C1q on phagosome maturation. Additionally, it would be very exciting to identify the C1q receptor(s) responsible for the functional effects observed in human cells. Given the clear role of C1q in avoiding human autoimmunity *in vivo*, the continued exploration of the effects of this extraordinary molecule will surely lead to novel and highly effective therapeutic modulation for SLE and other autoimmune diseases.

Reference List

Abbott RJ, Spendlove I, Roversi P, Fitzgibbon H, Knott V, Teriete P, McDonnell JM, Handford PA, Lea SM (2007) Structural and functional characterization of a novel T cell receptor co-regulatory protein complex, CD97-CD55. J Biol Chem 282:22023-22032.

Abdulahad DA, Westra J, Limburg PC, Kallenberg CG, Bijl M (2010) HMGB1 in systemic lupus Erythematosus: Its role in cutaneous lesions development. Autoimmun Rev 9:661-665.

Alexander JJ, Anderson AJ, Barnum SR, Stevens B, Tenner AJ (2008) The complement cascade: Yin-Yang in neuroinflammation--neuro-protection and -degeneration. J Neurochem 107:1169-1187.

Alper CA, Colten HR, Rosen FS, Rabson AR, Macnab GM, Gear JS (1972) Homozygous deficiency of C3 in a patient with repeated infections. Lancet 2:1179-1181.

Alunno A, Bartoloni E, Bistoni O, Nocentini G, Ronchetti S, Caterbi S, Valentini V, Riccardi C, Gerli R (2012) Balance between regulatory T and Th17 cells in systemic lupus erythematosus: the old and the new. Clin Dev Immunol 2012:823085.

Amarilyo G, Verbovetski I, Atallah M, Grau A, Wiser G, Gil O, Ben-Neriah Y, Mevorach D (2010) iC3b-opsonized apoptotic cells mediate a distinct anti-inflammatory response and transcriptional NF-kappaB-dependent blockade. Eur J Immunol 40:699-709.

Amel Kashipaz MR, Huggins ML, Powell RJ, Todd I (2002) Human autologous mixed lymphocyte reaction as an in vitro model for autoreactivity to apoptotic antigens. Immunology 107:358-365.

Anders HJ, Ryu M (2011) Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis. Kidney International 80:915-925.

Arvieux J, Yssel H, Colomb MG (1988) Antigen-bound C3b and C4b enhance antigen-presenting cell function in activation of human T-cell clones. Immunology 65:229-235.

Barchet W, Price JD, Cella M, Colonna M, MacMillan SK, Cobb JP, Thompson PA, Murphy KM, Atkinson JP, Kemper C (2006) Complement-induced regulatory T cells suppress T-cell responses but allow for dendritic-cell maturation. Blood 107:1497-1504.

Baruah P, Dumitriu IE, Malik TH, Cook HT, Dyson J, Scott D, Simpson E, Botto M (2009) C1q enhances IFN-gamma production by antigen-specific T cells via the CD40 costimulatory pathway on dendritic cells. Blood 113:3485-3493.

Baruah P, Simpson E, Dumitriu IE, Derbyshire K, Coe D, Addey C, Dyson J, Chai JG, Cook T, Scott D, Botto M (2010) Mice lacking C1q or C3 show accelerated rejection of minor H disparate skin grafts and resistance to induction of tolerance. Eur J Immunol 40:1758-1767.

Batten M, Li J, Yi S, Kljavin NM, Danilenko DM, Lucas S, Lee J, de Sauvage FJ, Ghilardi N (2006) Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. Nat Immunol 7:929-936.

Baudino L, Sardini A, Ruseva MM, Fossati-Jimack L, Cook HT, Scott D, Simpson E, Botto M (2014) C3 opsonization regulates endocytic handling of apoptotic cells resulting in enhanced T-cell responses to cargo-derived antigens. Proc Natl Acad Sci U S A 111:1503-1508.

Bell CW, Jiang W, Reich CF, III, Pisetsky DS (2006) The extracellular release of HMGB1 during apoptotic cell death. Am J Physiol Cell Physiol 291:C1318-C1325.

Benoit ME, Clarke EV, Morgado P, Fraser DA, Tenner AJ (2012) Complement protein C1q directs macrophage polarization and limits inflammasome activity during the uptake of apoptotic cells. J Immunol 188:5682-5693.

Benoit ME, Hernandez MX, Dinh ML, Benavente F, Vasquez O, Tenner AJ (2013) C1q-induced LRP1B and GPR6 Proteins Expressed Early in Alzheimer Disease Mouse Models, Are Essential for the C1q-mediated Protection against Amyloid-beta Neurotoxicity. J Biol Chem 288:654-665.

Benoit ME, Tenner AJ (2011) Complement protein C1q-mediated neuroprotection is correlated with regulation of neuronal gene and microRNA expression. J Neurosci 31:3459-3469.

Bensa JC, Reboul A, Colomb MG (1983) Biosynthesis in vitro of complement subcomponents C1q, C1s and C1 inhibitor by resting and stimulated human monocytes. Biochem J 216:385-392.

Beres AJ, Haribhai D, Chadwick AC, Gonyo PJ, Williams CB, Drobyski WR (2012) CD8+ Foxp3+ regulatory T cells are induced during graft-versus-host disease and mitigate disease severity. J Immunol 189:464-474.

Bialas AR, Stevens B (2013) TGF-beta signaling regulates neuronal C1q expression and developmental synaptic refinement. Nat Neurosci 16:1773-1782.

Bijl M, Reefman E, Horst G, Limburg PC, Kallenberg CG (2006) Reduced uptake of apoptotic cells by macrophages in systemic lupus erythematosus: correlates with decreased serum levels of complement. Ann Rheum Dis 65:57-63.

Biswas SK, Mantovani A (2010) Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol 11:889-896.

Bobak DA, Frank MM, Tenner AJ (1986) Characterization of C1q receptor expression on human phagocytic cells: Effects of PDBu and FMLP. J Immunol 136:4604-4610.

Bobak DA, Gaither TA, Frank MM, Tenner AJ (1987) Modulation of FcR function by complement: subcomponent C1q enhances the phagocytosis of IgG-opsonized targets by human monocytes and culture-derived macrophages. J Immunol 138:1150-1156.

Bode J, Dutow P, Sommer K, Janik K, Glage S, Tummler B, Munder A, Laudeley R, Sachse KW, Klos A (2012) A new role of the complement system: C3 provides protection in a mouse model of lung infection with intracellular Chlamydia psittaci. PLoS ONE 7:e50327.

Bohlson SS, Fraser DA, Tenner AJ (2007) Complement proteins C1q and MBL are pattern recognition molecules that signal immediate and long-term protective immune functions. Mol Immunol 44:33-43.

Bossi F, Tripodo C, Rizzi L, Bulla R, Agostinis C, Guarnotta C, Munaut C, Baldassarre G, Papa G, Zorzet S, Ghebrehiwet B, Ling GS, Botto M, Tedesco F (2014) C1q as a unique player in angiogenesis with therapeutic implication in wound healing. Proc Natl Acad Sci U S A 111:4209-4214.

Botto M, Dell'agnola C, Bygrave AE, Thompson EM, Cook HT, Petry F, Loos M, Pandolfi PP, Walport MJ (1998) Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat Genet 19:56-59.

Botto M, Walport MJ (2002) C1q, autoimmunity and apoptosis. Immunobiology 205:395-406.

Brazma A, et al. (2001) Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet 29:365-371.

Bretschneider I, Clemente MJ, Meisel C, Guerreiro M, Streitz M, Hopfenmuller W, Maciejewski JP, Wlodarski MW, Volk HD (2013) Discrimination of T-cell subsets and T-cell receptor repertoire distribution. Immunol Res 58:20-27.

Byrne JC, Ni GJ, Stacey KB, Coffey BM, McCarthy E, Thomas W, Jefferies CA (2013) Bruton's tyrosine kinase is required for apoptotic cell uptake via regulating the phosphorylation and localization of calreticulin. J Immunol 190:5207-5215.

Capasso M, Durrant LG, Stacey M, Gordon S, Ramage J, Spendlove I (2006) Costimulation via CD55 on human CD4+ T cells mediated by CD97. J Immunol 177:1070-1077.

Cardone J, Le FG, Vantourout P, Roberts A, Fuchs A, Jackson I, Suddason T, Lord G, Atkinson JP, Cope A, Hayday A, Kemper C (2010) Complement regulator CD46 temporally regulates cytokine production by conventional and unconventional T cells. Nat Immunol 11:862-871.

Carroll KC, Viollet B, Suttles J (2013) AMPKalpha1 deficiency amplifies proinflammatory myeloid APC activity and CD40 signaling. J Leukoc Biol 94:1113-1121.

Carroll MC, Isenman DE (2012) Regulation of humoral immunity by complement. Immunity 37:199-207.

Carter RH, Fearon DT (1992) CD19: Lowering the Threshold for Antigen Receptor Stimulation of B Lymphocytes. Science (New York, N Y) 256:105-107.

Castellano G, Trouw LA, Fiore N, Daha MR, Schena FP, van KC (2010) Infiltrating dendritic cells contribute to local synthesis of C1q in murine and human lupus nephritis. Mol Immunol 47:2129-2137.

Castellano G, Woltman AM, Nauta AJ, Roos A, Trouw LA, Seelen MA, Schena FP, Daha MR, van Kooten C (2004a) Maturation of dendritic cells abrogates C1q production in vivo and in vitro. Blood 103:3813-3820.

Castellano G, Woltman AM, Schena FP, Roos A, Daha MR, van Kooten C (2004b) Dendritic cells and complement: at the cross road of innate and adaptive immunity. Mol Immunol 41:133-140.

Castellano G, Woltman AM, Schlagwein N, Xu W, Schena FP, Daha MR, van KC (2007) Immune modulation of human dendritic cells by complement. Eur J Immunol 37:2803-2811.

Caux C, Vanbervliet B, Massacrier C, Azuma M, Okumura K, Lanier LL, Banchereau J (1994) B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. J Exp Med 180:1841-1847.

Chatzigeorgiou A, Lyberi M, Chatzilymperis G, Nezos A, Kamper E (2009) CD40/CD40L signaling and its implication in health and disease. Biofactors 35:474-483.

Chen A, Gaddipati S, Hong Y, Volkman DJ, Peerschke EIB, Ghebrehiwet B (1994) Human T cells express specific binding sites for C1q: Role in T cell activation and proliferation. J Immunol 153:1430-1440.

Chernysheva AD, Kirou KA, Crow MK (2002) T cell proliferation induced by autologous non-T cells is a response to apoptotic cells processed by dendritic cells. J Immunol 169:1241-1250.

Christmas SE, de la Mata Espinosa CT, Halliday D, Buxton CA, Cummerson JA, Johnson PM (2006) Levels of expression of complement regulatory proteins CD46, CD55 and CD59 on resting and activated human peripheral blood leucocytes. Immunology 119:522-528.

Clarke EV, Fraser DA, Benoit ME, Tenner AJ (2011) C1q Activates Distinct Signaling Mechanisms in Human Monocytes, Macrophages and Immature Dendritic cells. J Leukoc Biol Supplement1-48.

Cline MS, et al. (2007) Integration of biological networks and gene expression data using Cytoscape. Nat Protoc 2:2366-2382.

Cretney E, Kallies A, Nutt SL (2013) Differentiation and function of Foxp3(+) effector regulatory T cells. Trends Immunol 34:74-80.

Crispin JC, Tsokos GC (2010) Interleukin-17-producing T cells in lupus. Curr Opin Rheumatol 22:499-503.

Cutler AJ, Botto M, van ED, Rivi R, Davies KA, Gray D, Walport MJ (1998) T cell-dependent immune response in C1q-deficient mice: defective interferon gamma production by antigen-specific T cells. J Exp Med 187:1789-1797.

Delamarre L, Couture R, Mellman I, Trombetta ES (2006) Enhancing immunogenicity by limiting susceptibility to lysosomal proteolysis. J Exp Med 203:2049-2055.

Dietzschold B, Schwaeble W, Schafer MK, Hooper DC, Zehng YM, Petry F, Sheng H, Fink T, Loos M, Koprowski H, Weihe E (1995) Expression of C1q, a subcomponent of the rat complement system, is dramatically enhanced in brains of rats with either Borna disease or experimental allergic encephalomyelitis. J Neurol Sci 130:11-16.

Ding Y, Li J, Wu Q, Yang P, Luo B, Xie S, Druey KM, Zajac AJ, Hsu HC, Mountz JD (2013) IL-17RA is essential for optimal localization of follicular Th cells in the germinal center light zone to promote autoantibody-producing B cells. J Immunol 191:1614-1624.

Diveu C, McGeachy MJ, Boniface K, Stumhofer JS, Sathe M, Joyce-Shaikh B, Chen Y, Tato CM, McClanahan TK, de Waal MR, Hunter CA, Cua DJ, Kastelein RA (2009) IL-27 blocks RORc expression to inhibit lineage commitment of Th17 cells. J Immunol 182:5748-5756.

Dunkelberger J, Zhou L, Miwa T, Song WC (2012) C5aR expression in a novel GFP reporter gene knockin mouse: implications for the mechanism of action of C5aR signaling in T cell immunity. J Immunol 188:4032-4042.

Dunkelberger JR, Song WC (2010) Role and mechanism of action of complement in regulating T cell immunity. Mol Immunol 47:2176-2186.

Dunne A, Ross PJ, Pospisilova E, Masin J, Meaney A, Sutton CE, Iwakura Y, Tschopp J, Sebo P, Mills KH (2010) Inflammasome activation by adenylate cyclase toxin directs Th17 responses and protection against Bordetella pertussis. J Immunol 185:1711-1719.

Dutow P, Fehlhaber B, Bode J, Laudeley R, Rheinheimer C, Glage S, Wetsel RA, Pabst O, Klos A (2014) The Complement C3a Receptor Is Critical in Defense against Chlamydia psittaci in Mouse Lung Infection and Required for Antibody and Optimal T Cell Response. J Infect Dis 209:1269-1278.

Duus K, Hansen EW, Tacnet P, Frachet P, Arlaud GrJ, Thielens NM, Houen G (2010) Direct interaction between CD91 and C1q. The FEBS journal 277:3526-3537.

Elkon KB, Santer DM (2012) Complement, interferon and lupus. Curr Opin Immunol 24:665-670.

Erwig LP, Henson PM (2008) Clearance of apoptotic cells by phagocytes. Cell Death Differ 15:243-250.

Fang C, Miwa T, Shen H, Song WC (2007) Complement-dependent enhancement of CD8+ T cell immunity to lymphocytic choriomeningitis virus infection in decay-accelerating factor-deficient mice. J Immunol 179:3178-3186.

Fang C, Miwa T, Song WC (2011) Decay-accelerating factor regulates T-cell immunity in the context of inflammation by influencing costimulatory molecule expression on antigen-presenting cells. Blood 118:1008-1014.

Feng X, Wu H, Grossman JM, Hanvivadhanakul P, FitzGerald JD, Park GS, Dong X, Chen W, Kim MH, Weng HH, Furst DE, Gorn A, McMahon M, Taylor M, Brahn E, Hahn BH, Tsao BP (2006) Association of increased interferon-inducible gene expression with disease activity and lupus nephritis in patients with systemic lupus erythematosus. Arthritis Rheum 54:2951-2962.

Fife BT, Pauken KE, Eagar TN, Obu T, Wu J, Tang Q, Azuma M, Krummel MF, Bluestone JA (2009) Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. Nat Immunol 10:1185-1192.

Fraser DA, Arora M, Bohlson SS, Lozano E, Tenner AJ (2007) Generation of inhibitory NF{kappa}B complexes and phosphorylated cAMP response element-binding protein correlates with the activity of complement protein C1q in human monocytes. J Biol Chem 282:7360-7367.

Fraser DA, Bohlson SS, Jasinskiene N, Rawal N, Palmarini G, Ruiz S, Rochford R, Tenner AJ (2006) C1q and MBL, components of the innate immune system, influence monocyte cytokine expression. J Leukoc Biol 80:107-116.

Fraser DA, Laust AK, Nelson EL, Tenner AJ (2009) C1q differentially modulates phagocytosis and cytokine responses during ingestion of apoptotic cells by human monocytes, macrophages, and dendritic cells. J Immunol 183:6175-6185.

Fraser DA, Pisalyaput K, Tenner AJ (2010) C1q enhances microglial clearance of apoptotic neurons and neuronal blebs, and modulates subsequent inflammatory cytokine production. J Neurochem 112:733-743.

Fraser DA, Tenner AJ (2008) Directing an appropriate immune response: the role of defense collagens and other soluble pattern recognition molecules. Curr Drug Targets 9:113-122.

Fraser DA, Tenner AJ (2010) Innate immune proteins C1q and mannan-binding lectin enhance clearance of atherogenic lipoproteins by human monocytes and macrophages. J Immunol 185:3932-3939.

Fremeaux-Bacchi V, Moulton EA, Kavanagh D, Dragon-Durey MA, Blouin J, Caudy A, Arzouk N, Cleper R, Francois M, Guest G, Pourrat J, Seligman R, Fridman WH, Loirat C, Atkinson JP (2006) Genetic and functional analyses of membrane cofactor protein (CD46) mutations in atypical hemolytic uremic syndrome. J Am Soc Nephrol 17:2017-2025.

Galvan MD, Foreman DB, Zeng E, Tan JC, Bohlson SS (2012a) Complement component C1q regulates macrophage expression of Mer tyrosine kinase to promote clearance of apoptotic cells. J Immunol 188:3716-3723.

Galvan MD, Foreman DB, Zeng E, Tan JC, Bohlson SS (2012b) Complement Component C1q Regulates Macrophage Expression of Mer Tyrosine Kinase To Promote Clearance of Apoptotic Cells. J Immunol.

Galvan MD, Hulsebus H, Heitker T, Zeng E, Bohlson SS (2014) Complement Protein C1q and Adiponectin Stimulate Mer Tyrosine Kinase-Dependent Engulfment of Apoptotic Cells through a Shared Pathway. J Innate Immun.

Gao X, Liu H, Ding G, Wang Z, Fu H, Ni Z, Ma J, Liu F, Fu Z (2011) Complement C3 deficiency prevent against the onset of streptozotocin-induced autoimmune diabetes involving expansion of regulatory T cells. Clin Immunol 140:236-243.

Garcia-Valladares I, Atisha-Fregoso Y, Richaud-Patin Y, Jakez-Ocampo J, Soto-Vega E, Elias-Lopez D, Carrillo-Maravilla E, Cabiedes J, Ruiz-Arguelles A, Llorente L (2006) Diminished expression of complement regulatory proteins (CD55 and CD59) in lymphocytes from systemic lupus erythematosus patients with lymphopenia. Lupus 15:600-605.

Ghai R, Waters P, Roumenina LT, Gadjeva M, Kojouharova MS, Reid KB, Sim RB, Kishore U (2007) C1q and its growing family. Immunobiology 212:253-266.

Ghannam A, Fauquert JL, Thomas C, Kemper C, Drouet C (2014) Human complement C3 deficiency: Th1 induction requires T cell-derived complement C3a and CD46 activation. Mol Immunol 58:98-107.

Ghannam A, Pernollet M, Fauquert JL, Monnier N, Ponard D, Villiers MB, Peguet-Navarro J, Tridon A, Lunardi J, Gerlier D, Drouet C (2008) Human C3 deficiency associated with impairments in dendritic cell differentiation, memory B cells, and regulatory T cells. J Immunol 181:5158-5166.

Ghoreschi K, Laurence A, Yang XP, Hirahara K, O'Shea JJ (2011) T helper 17 cell heterogeneity and pathogenicity in autoimmune disease. Trends Immunol 32:395-401.

Goldsmith SK, Wals P, Rozovsky I, Morgan TE, Finch CE (1997) Kainic acid and decorticating lesions stimulate the synthesis of C1q protein in adult rat brain. J of Neurochemistry 68:2046-2052.

Gompels MM, Lock RJ, Abinun M, Bethune CA, Davies G, Grattan C, Fay AC, Longhurst HJ, Morrison L, Price A, Price M, Watters D (2005) C1 inhibitor deficiency: consensus document. Clinical and Experimental Immunology 139:379-394.

Guan E, Burgess WH, Robinson SL, Goodman EB, McTigue KJ, Tenner AJ (1991) Phagocytic cell molecules that bind the collagen-like region of C1q: Involvement in the C1q-mediated enhancement of phagocytosis. J Biol Chem 266:20345-20355.

Guarda G, Braun M, Staehli F, Tardivel A, Mattmann C, Forster I, Farlik M, Decker T, Du Pasquier RA, Romero P, Tschopp J (2011) Type I interferon inhibits interleukin-1 production and inflammasome activation. Immunity 34:213-223.

Gullstrand B, Martensson U, Sturfelt G, Bengtsson AA, Truedsson L (2009) Complement classical pathway components are all important in clearance of apoptotic and secondary necrotic cells. Clin Exp Immunol 156:303-311.

Hardie DG (2007) AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nature reviews Molecular cell biology 8:774-785.

Harley JB, et al. (2008) Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. Nat Genet 40:204-210.

Hashimoto M, Hirota K, Yoshitomi H, Maeda S, Teradaira S, Akizuki S, Prieto-Martin P, Nomura T, Sakaguchi N, Kohl J, Heyman B, Takahashi M, Fujita T, Mimori T, Sakaguchi S (2010) Complement drives Th17 cell differentiation and triggers autoimmune arthritis. J Exp Med 207:1135-1143.

Heeger PS, Kemper C (2012) Novel roles of complement in T effector cell regulation. Immunobiology 217:216-224.

Hirahara K, Ghoreschi K, Yang XP, Takahashi H, Laurence A, Vahedi G, Sciume G, Hall AO, Dupont CD, Francisco LM, Chen Q, Tanaka M, Kanno Y, Sun HW, Sharpe AH, Hunter CA, O'Shea JJ (2012) Interleukin-27 priming of T cells controls IL-17 production in trans via induction of the ligand PD-L1. Immunity 36:1017-1030.

Hosszu KK, Valentino A, Vinayagasundaram U, Vinayagasundaram R, Joyce MG, Ji Y, Peerschke EI, Ghebrehiwet B (2012) DC-SIGN, C1q, and gC1qR form a trimolecular receptor complex on the surface of monocyte-derived immature dendritic cells. Blood 120:1228-1236.

Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM (1993) Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science (New York, N Y) 260:547-549.

Huang dW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44-57.

Huber S, Hoffmann R, Muskens F, Voehringer D (2010) Alternatively activated macrophages inhibit T-cell proliferation by Stat6-dependent expression of PD-L2. Blood 116:3311-3320.

Ito S, Ansari P, Sakatsume M, Dickensheets H, Vazquez N, Donnelly RP, Larner AC, Finbloom DS (1999) Interleukin-10 inhibits expression of both interferon alpha- and interferon gamma- induced genes by suppressing tyrosine phosphorylation of STAT1. Blood 93:1456-1463.

Iyer SS, Ghaffari AA, Cheng G (2010) Lipopolysaccharide-mediated IL-10 transcriptional regulation requires sequential induction of type I IFNs and IL-27 in macrophages. J Immunol 185:6599-6607.

Jetten N, Verbruggen S, Gijbels MJ, Post MJ, de Winther MP, Donners MM (2014) Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo. Angiogenesis 17:109-118.

Kang HK, Liu M, Datta SK (2007) Low-dose peptide tolerance therapy of lupus generates plasmacytoid dendritic cells that cause expansion of autoantigen-specific regulatory T cells and contraction of inflammatory Th17 cells. J Immunol 178:7849-7858.

Karakhanova S, Bedke T, Enk AH, Mahnke K (2011) IL-27 renders DC immunosuppressive by induction of B7-H1. J Leukoc Biol 89:837-845.

Karp CL, Wysocka M, Wahl LM, Ahearn JM, Cuomo PJ, Sherry B, Trinchieri G, Griffin DE (1996) Mechanism of suppression of cell-mediated immunity by measles virus. Science (New York, N Y) 273:228-231.

Kelley J, Johnson MR, Alarcon GS, Kimberly RP, Edberg JC (2007) Variation in the relative copy number of the TLR7 gene in patients with systemic lupus erythematosus and healthy control subjects. Arthritis Rheum 56:3375-3378.

Kemper C, Chan AC, Green JM, Brett KA, Murphy KM, Atkinson JP (2003) Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. Nature 421:388-392.

Kemper C, Kohl J (2013) Novel roles for complement receptors in T cell regulation and beyond. Mol Immunol 56:181-190.

Kemper C, Mitchell LM, Zhang L, Hourcade DE (2008) The complement protein properdin binds apoptotic T cells and promotes complement activation and phagocytosis. Proc Natl Acad Sci U S A 105:9023-9028.

Kerekes K, Cooper PD, Prechl J, Jozsi M, Bajtay Z, Erdei A (2001) Adjuvant effect of gamma-inulin is mediated by C3 fragments deposited on antigen-presenting cells. J Leukoc Biol 69:69-74.

Khoury MK, Parker I, Aswad DW (2010) Acquisition of chemiluminescent signals from immunoblots with a digital single-lens reflex camera. Anal Biochem 397:129-131.

Kido M, Takeuchi S, Sugiyama N, Esaki H, Nakashima H, Yoshida H, Furue M (2011) T cell-specific overexpression of interleukin-27 receptor +¦ subunit (WSX-1) prevents spontaneous skin inflammation in MRL/lpr mice. The British journal of dermatology 164:1214-1220.

Kim HJ, Hart J, Knatz N, Hall MW, Wewers MD (2004) Janus kinase 3 down-regulates lipopolysaccharide-induced IL-1 beta-converting enzyme activation by autocrine IL-10. J Immunol 172:4948-4955.

Kim HJ, Verbinnen B, Tang X, Lu L, Cantor H (2010) Inhibition of follicular T-helper cells by CD8(+) regulatory T cells is essential for self tolerance. Nature 467:328-332.

Kinchen JM, Ravichandran KS (2008) Phagosome maturation: going through the acid test. Nat Rev Mol Cell Biol 9:781-795.

Kinoshita T, Medof ME, Silber R, Nussenzweig V (1985) Distribution of decay-accelerating factor in the peripheral blood of normal individuals and patients with paroxysmal nocturnal hemoglobinuria. J Exp Med 162:75-92.

Klickstein LB, Barbashov SF, Liu T, Jack RM, Nicholson-Weller A (1997) Complement receptor type 1 (CR1, CD35) is a receptor for C1q. Immunity 7:345-355.

Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, Kohl J (2009) The role of the anaphylatoxins in health and disease. Mol Immunol 46:2753-2766.

Kono H, Rock KL (2008) How dying cells alert the immune system to danger. Nat Rev Immunol 8:279-289.

Korb LC, Ahearn JM (1997) C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. J Immunol 158:4525-4528.

Krausgruber T, Blazek K, Smallie T, Alzabin S, Lockstone H, Sahgal N, Hussell T, Feldmann M, Udalova IA (2011) IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. Nature Publishing Group 12:231-238.

Krieg AM, Vollmer J (2007) Toll-like receptors 7, 8, and 9: linking innate immunity to autoimmunity. Immunol Rev 220:251-269.

Kurowska-Stolarska M, Stolarski B, Kewin P, Murphy G, Corrigan CJ, Ying S, Pitman N, Mirchandani A, Rana B, van RN, Shepherd M, McSharry C, McInnes IB, Xu D, Liew FY (2009) IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. j immunol 183:6469-6477.

Kwan WH, Hashimoto D, Paz-Artal E, Ostrow K, Greter M, Raedler H, Medof ME, Merad M, Heeger PS (2012) Antigen-presenting cell-derived complement modulates graft-versus-host disease. J Clin Invest 122:2234-2238.

Kwan WH, van der Touw W, Paz-Artal E, Li MO, Heeger PS (2013) Signaling through C5a receptor and C3a receptor diminishes function of murine natural regulatory T cells. J Exp Med 210:257-268.

Lajoie S, Lewkowich IP, Suzuki Y, Clark JR, Sproles AA, Dienger K, Budelsky AL, Wills-Karp M (2010) Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. Nat Immunol 11:928-935.

Latchman Y, et al. (2001) PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2:261-268.

Lazarevic V, Chen X, Shim JH, Hwang ES, Jang E, Bolm AN, Oukka M, Kuchroo VK, Glimcher LH (2011) T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORgammat. Nat Immunol 12:96-104.

LeBuanec H., Gougeon ML, Mathian A, Lebon P, Dupont JM, Peltre G, Hemon P, Schmid M, Bizzini B, Kunding T, Burny A, Bensussan A, Amoura Z, Gallo RC, Zagury D (2011) IFN-alpha and CD46 stimulation are associated with active lupus and skew natural T regulatory cell differentiation to type 1 regulatory T (Tr1) cells. Proc Natl Acad Sci U S A 108:18995-19000.

Lech M, Grobmayr R, Weidenbusch M, Anders HJ (2012) Tissues use resident dendritic cells and macrophages to maintain homeostasis and to regain homeostasis upon tissue injury: the immunoregulatory role of changing tissue environments. Mediators Inflamm 2012:951390.

LeFriec G., Kohl J, Kemper C (2013) A complement a day keeps the Fox(p3) away. Nat Immunol 14:110-112.

LeFriec G., et al. (2012) The CD46-Jagged1 interaction is critical for human TH1 immunity. Nat Immunol 13:1213-1221.

Lichtenegger FS, Mueller K, Otte B, Beck B, Hiddemann W, Schendel DJ, Subklewe M (2012) CD86 and IL-12p70 are key players for T helper 1 polarization and natural killer cell activation by Toll-like receptor-induced dendritic cells. PLoS ONE 7:e44266.

Lionetti FJ, Hunt SM, Valeri CR (1980) Methods of Cell Separation. New York: Plenum Publishing Corp.

Liszewski MK, Kolev M, Le FG, Leung M, Bertram PG, Fara AF, Subias M, Pickering MC, Drouet C, Meri S, Arstila TP, Pekkarinen PT, Ma M, Cope A, Reinheckel T, Rodriguez de CS, Afzali B, Atkinson JP, Kemper C (2013) Intracellular complement activation sustains T cell homeostasis and mediates effector differentiation. Immunity 39:1143-1157.

Liszewski MK, Post TW, Atkinson JP (1991) Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. Annu Rev Immunol 9:431-455.

Liu J, Miwa T, Hilliard B, Chen Y, Lambris JD, Wells AD, Song WC (2005) The complement inhibitory protein DAF (CD55) suppresses T cell immunity in vivo. J Exp Med 201:567-577.

Liu T, Xu G, Guo B, Fu Y, Qiu Y, Ding Y, Zheng H, Fu X, Wu Y, Xu W (2013) An essential role for C5aR signaling in the optimal induction of a malaria-specific CD4+ T cell response by a whole-killed blood-stage vaccine. J Immunol 191:178-186.

Liu Z, Davidson A (2012) Taming lupus—a new understanding of pathogenesis is leading to clinical advances. Nature Publishing Group 18:871-882.

Lu JH, Teh BK, Wang Ld, Wang YN, Tan YS, Lai MC, Reid KBM (2008) The classical and regulatory functions of C1q in immunity and autoimmunity. Cellular & molecular immunology 5:9-21.

Lundberg K, Rydnert F, Greiff L, Lindstedt M (2014) Human blood dendritic cell subsets exhibit discriminative pattern recognition receptor profiles. Immunology 142:279-288.

Ma Q, Li D, Nurieva R, Patenia R, Bassett R, Cao W, Alekseev AM, He H, Molldrem JJ, Kroll MH, Champlin RE, Sale GE, Afshar-Kharghan V (2012a) Reduced graft-versus-host disease in C3-deficient mice is associated with decreased donor Th1/Th17 differentiation. Biol Blood Marrow Transplant 18:1174-1181.

Ma W, Rai V, Hudson BI, Song F, Schmidt AM, Barile GR (2012b) RAGE binds C1q and enhances C1q-mediated phagocytosis. Cell Immunol 274:72-82.

Maecker HT, McCoy JP, Nussenblatt R (2012) Standardizing immunophenotyping for the Human Immunology Project. Nature Reviews Immunology 12:191-200.

Majai G, Gogolak P, Ambrus C, Vereb G, Hodrea J, Fesus L, Rajnavolgyi E (2010) PPARgamma modulated inflammatory response of human dendritic cell subsets to engulfed apoptotic neutrophils. J Leukoc Biol 88:981-991.

Malhotra R, Thiel S, Reid KBM, Sim RB (1990) Human Leukocyte C1q Receptor Binds Other Soluble Proteins with Collagen Domains. J Exp Med 172:955-959.

Mallone R, Mannering SI, Brooks-Worrell BM, Durinovic-Bello I, Cilio CM, Wong FS, Schloot NC (2011) Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. Clin exp Immunol 163:33-49.

Manderson AP, Botto M, Walport MJ (2004) The role of complement in the development of systemic lupus erythematosus. Annu Rev Immunol 22:431-456.

Martens HA, Zuurman MW, de Lange AH, Nolte IM, van der Steege G, Navis GJ, Kallenberg CG, Seelen MA, Bijl M (2009) Analysis of C1q polymorphisms suggests association with systemic lupus erythematosus, serum C1q and CH50 levels and disease severity. Ann Rheum Dis 68:715-720.

Martin U, Bock D, Arseniev L, Tornetta MA, Ames RS, Bautsch W, Kohl J, Ganser A, Klos A (1997) The human C3a receptor is expressed on neutrophils and monocytes, but not on B or T lymphocytes. J Exp Med 186:199-207.

Martinez FO, Sica A, Mantovani A, Locati M (2008) Macrophage activation and polarization. Frontiers in bioscience : a journal and virtual library 13:453-461.

Mascanfroni ID, Yeste A, Vieira SM, Burns EJ, Patel B, Sloma I, Wu Y, Mayo L, Ben-Hamo R, Efroni S, Kuchroo VK, Robson SC, Quintana FJ (2013) IL-27 acts on DCs to suppress the T cell response and autoimmunity by inducing expression of the immunoregulatory molecule CD39. Nat Immunol 14:1054-1063.

McFarland BC, Gray GK, Nozell SE, Hong SW, Benveniste EN (2013) Activation of the NF-kappaB pathway by the STAT3 inhibitor JSI-124 in human glioblastoma cells. Mol Cancer Res 11:494-505.

Merad M, Sathe P, Helft J, Miller J, Mortha A (2013) The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol 31:563-604.

Mestas J, Hughes CC (2004) Of mice and not men: differences between mouse and human immunology. J Immunol 172:2731-2738.

Mitchell DA, Pickering MC, Warren J, Fossati-Jimack L, Cortes-Hernandez J, Cook HT, Botto M, Walport MJ (2002) C1q deficiency and autoimmunity: the effects of genetic background on disease expression. J Immunol 168:2538-2543.

Mittag D, Proietto AI, Loudovaris T, Mannering SI, Vremec D, Shortman K, Wu L, Harrison LC (2011) Human dendritic cell subsets from spleen and blood are similar in phenotype and function but modified by donor health status. J Immunol 186:6207-6217.

Miura-Shimura Y, Nakamura K, Ohtsuji M, Tomita H, Jiang Y, Abe M, Zhang D, Hamano Y, Tsuda H, Hashimoto H, Nishimura H, Taki S, Shirai T, Hirose S (2002) C1q regulatory region polymorphism down-regulating murine C1q protein levels with linkage to lupus nephritis. J Immunol 169:1334-1339.

Miyake K, Akahoshi M, Nakashima H (2011) Th subset balance in lupus nephritis. J Biomed Biotechnol 2011:980286.

Mosser DM, Zhang X (2008) Interleukin-10: new perspectives on an old cytokine. Immunol Rev 226:205-218.

Mukherjee S, Giamberardino C, Thomas JM, Gowdy K, Pastva AM, Wright JR (2012) Surfactant protein A modulates induction of regulatory T cells via TGF-beta. J Immunol 188:4376-4384.

Mukundan L, Odegaard JI, Morel CR, Heredia JE, Mwangi JW, Ricardo-Gonzalez RR, Goh YP, Eagle AR, Dunn SE, Awakuni JU, Nguyen KD, Steinman L, Michie SA, Chawla A (2009) PPAR-delta senses and orchestrates clearance of apoptotic cells to promote tolerance. Nat Med 15:1266-1272.

Murray PJ (2007) The JAK-STAT signaling pathway: input and output integration. J Immunol 178:2623-2629.

Nakano Y, Morita S, Kawamoto A, Suda T, Chida K, Nakamura H (2003) Elevated complement C3a in plasma from patients with severe acute asthma. J Allergy Clin Immunol 112:525-530.

Nakayama Y, Kim SI, Kim EH, Lambris JD, Sandor M, Suresh M (2009) C3 promotes expansion of CD8+ and CD4+ T cells in a Listeria monocytogenes infection. J Immunol 183:2921-2931.

Nataf S, Davoust N, Ames RS, Barnum SR (1999) Human T cells express the C5a receptor and are chemoattracted to C5a. J Immunol 162:4018-4023.

Nauta AJ, Castellano G, Xu W, Woltman AM, Borrias MC, Daha MR, van Kooten C, Roos A (2004) Opsonization with C1q and mannose-binding lectin targets apoptotic cells to dendritic cells. J Immunol 173:3044-3050.

Navratil JS, Watkins SC, Wisnieski JJ, Ahearn JM (2001) The globular heads of C1q specifically recognize surface blebs of apoptotic vascular endothelial cells. J Immunol 166:3231-3239.

Nicholson-Weller A, March JP, Rosenfeld SI, AUSTEN KF (1983) Affected erythrocytes of patients with paroxysmal nocturnal hemoglobinuria are deficient in the complement regulatory protein, decay accelerating factor. Proc Natl Acad Sci U S A 80:5066-5070.

Nold MF, Nold-Petry CA, Zepp JA, Palmer BE, Bufler P, Dinarello CA (2010) IL-37 is a fundamental inhibitor of innate immunity. Nat Immunol 11:1014-1022.

Norsworthy PJ, Fossati-Jimack L, Cortes-Hernandez J, Taylor PR, Bygrave AE, Thompson RD, Nourshargh S, Walport MJ, Botto M (2004) Murine CD93 (C1qRp) contributes to the removal of apoptotic cells in vivo but is not required for C1q-mediated enhancement of phagocytosis. J Immunol 172:3406-3414.

O'Shea JJ, Paul WE (2010) Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. Science (New York, N Y) 327:1098-1102.

Ogden CA, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, Henson PM (2001) C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J Exp Med 194:781-796.

Osterburg AR, Hexley P, Supp DM, Robinson CT, Noel G, Ogle C, Boyce ST, Aronow BJ, Babcock GF (2013) Concerns over interspecies transcriptional comparisons in mice and humans after trauma. Proc Natl Acad Sci U S A 110:E3370.

Paidassi H, Tacnet-Delorme P, Verneret M+, Gaboriaud C, Houen G, Duus K, Ling WL, Arlaud GJ, Frachet P (2011) Investigations on the C1q-calreticulin-phosphatidylserine interactions yield new insights into apoptotic cell recognition. J Mol Biol 408:277-290.

Pavlov V, Raedler H, Yuan S, Leisman S, Kwan WH, Lalli PN, Medof ME, Heeger PS (2008) Donor deficiency of decay-accelerating factor accelerates murine T cell-mediated cardiac allograft rejection. J Immunol 181:4580-4589.

Peng X, Kasran A, Warmerdam PA, de BM, Ceuppens JL (1996) Accessory signaling by CD40 for T cell activation: induction of Th1 and Th2 cytokines and synergy with interleukin-12 for interferon-gamma production. Eur J Immunol 26:1621-1627.

Petry F, Reid KBM, Loos M (1991) Gene expression of the A- and B-chain of mouse C1q in different tissues and the characterization of the recombinant A-chain. J Immunol 147:3988-3993.

Pickering MC, Botto M, Taylor PR, Lachmann PJ, Walport MJ (2000) Systemic lupus erythematosus, complement deficiency, and apoptosis. Adv Immunol 76:227-324.

Piganis RAR, De Weerd NA, Gould JA, Schindler CW, Mansell A, Nicholson SE, Hertzog PJ (2011) Suppressor of cytokine signaling (SOCS) 1 inhibits type I interferon (IFN) signaling via the interferon alpha receptor (IFNAR1)-associated tyrosine kinase Tyk2. J Biol Chem 286:33811-33818.

Pisitkun P, Ha HL, Wang H, Claudio E, Tivy CC, Zhou H, Mayadas TN, Illei GG, Siebenlist U (2012) Interleukin-17 cytokines are critical in development of fatal lupus glomerulonephritis. Immunity 37:1104-1115.

Potter PK, Cortes-Hernandez J, Quartier P, Botto M, Walport MJ (2003) Lupus-prone mice have an abnormal response to thioglycolate and an impaired clearance of apoptotic cells. J Immunol 170:3223-3232.

Ramirez-Ortiz ZG, Pendergraft WF, III, Prasad A, Byrne MH, Iram T, Blanchette CJ, Luster AD, Hacohen N, Khoury JE, Means TK (2013) The scavenger receptor SCARF1 mediates the clearance of apoptotic cells and prevents autoimmunity. Nat Immunol 14:917-926.

Rathmell JC, Farkash EA, Gao W, Thompson CB (2001) IL-7 enhances the survival and maintains the size of naive T cells. J Immunol 167:6869-6876.

Ricklin D, Hajishengallis G, Yang K, Lambris JD (2010) Complement: a key system for immune surveillance and homeostasis. Nat Immunol 11:785-797.

Ricklin D, Lambris JD (2013) Complement in immune and inflammatory disorders: pathophysiological mechanisms. J Immunol 190:3831-3838.

Rock KL, Kono H (2008) The inflammatory response to cell death. Annu Rev Pathol 3:99-126.

Rosenfeld SI, Kelly ME, Leddy JP (1976) Hereditary deficiency of the fifth component of complement in man. I. Clinical, immunochemical, and family studies. J Clin Invest 57:1626-1634.

Roszer T, Menendez-Gutierrez MP, Lefterova MI, Alameda D, Nunez V, Lazar MA, Fischer T, Ricote M (2011) Autoimmune kidney disease and impaired engulfment of apoptotic cells in mice with macrophage peroxisome proliferator-activated receptor gamma or retinoid X receptor alpha deficiency. j immunol 186:621-631.

Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, Li J, Thiagarajan M, White JA, Quackenbush J (2006) TM4 microarray software suite. Methods Enzymol 411:134-193.

Sag D, Carling D, Stout RD, Suttles J (2008) Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. j immunol 181:8633-8641.

Sakaguchi S, Yamaguchi T, Nomura T, Ono M (2008) Regulatory T cells and immune tolerance. Cell 133:775-787.

Sallusto F, Geginat J, Lanzavecchia A (2004) Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 22:745-763.

Santer DM, Hall BE, George TC, Tangsombatvisit S, Liu CL, Arkwright PD, Elkon KB (2010) C1q deficiency leads to the defective suppression of IFN-alpha in response to nucleoprotein containing immune complexes. J Immunol 185:4738-4749.

Santer DM, Wiedeman AE, Teal TH, Ghosh P, Elkon KB (2012) Plasmacytoid dendritic cells and C1q differentially regulate inflammatory gene induction by lupus immune complexes. J Immunol 188:902-915.

Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJ, Bickham KL, Lerner H, Goldstein M, Sykes M, Kato T, Farber DL (2013) Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. Immunity 38:187-197.

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101-1108.

Schmudde I, Strover HA, Vollbrandt T, Konig P, Karsten CM, Laumonnier Y, Kohl J (2013) C5a receptor signalling in dendritic cells controls the development of maladaptive Th2 and Th17 immunity in experimental allergic asthma. Mucosal Immunol 6:807-825.

Seok J, et al. (2013) Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A 110:3507-3512.

Serra VA, Cretin F, Pepin E, Gabert FM, Marche PN (1997) Complement C3b fragment covalently linked to tetanus toxin increases lysosomal sodium dodecyl sulfate-stable HLA-DR dimer production. Eur J Immunol 27:2673-2679.

Shen N, et al. (2010) Sex-specific association of X-linked Toll-like receptor 7 (TLR7) with male systemic lupus erythematosus. Proc Natl Acad Sci U S A 107:15838-15843.

Shin MS, Lee N, Kang I (2011) Effector T-cell subsets in systemic lupus erythematosus: update focusing on Th17 cells. Curr Opin Rheumatol 23:444-448.

Singer L, Colten HR, Wetsel RA (1994) Complement C3 deficiency: human, animal, and experimental models. Pathobiology 62:14-28.

Sjoberg AP, Trouw LA, Blom AM (2009) Complement activation and inhibition: a delicate balance. Trends Immunol 30:83-90.

Skapenko A, Leipe J, Lipsky PE, Schulze-Koops H (2005) The role of the T cell in autoimmune inflammation. Arthritis Res Ther 7 Suppl 2:S4-14.

Son M, Santiago-Schwarz F, Al-Abed Y, Diamond B (2012) C1q limits dendritic cell differentiation and activation by engaging LAIR-1. Proc Natl Acad Sci U S A 109:E3160-E3167.

Sontheimer RD, Racila E, Racila DM (2005) C1q: Its Functions within the Innate and Adaptive Immune Responses and its Role in Lupus Autoimmunity. J Invest Dermatol 125:14-23.

Stehlik C, Dorfleutner A (2007) COPs and POPs: modulators of inflammasome activity. J Immunol 179:7993-7998.

Stehlik C, Krajewska M, Welsh K, Krajewski S, Godzik A, Reed JC (2003) The PAAD/PYRIN-only protein POP1/ASC2 is a modulator of ASC-mediated nuclear-factor-kappa B and pro-caspase-1 regulation. Biochem J 373:101-113.

Strainic MG, Liu J, Huang D, An F, Lalli PN, Muqim N, Shapiro VS, Dubyak GR, Heeger PS, Medof ME (2008) Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. Immunity 28:425-435.

Strainic MG, Shevach EM, An F, Lin F, Medof ME (2013) Absence of signaling into CD4(+) cells via C3aR and C5aR enables autoinductive TGF-beta1 signaling and induction of Foxp3(+) regulatory T cells. Nat Immunol 14:162-171.

Stumhofer JS, Hunter CA (2008) Advances in understanding the anti-inflammatory properties of IL-27. Immunol Let 117:123-130.

Sugiyama N, Nakashima H, Yoshimura T, Sadanaga A, Shimizu S, Masutani K, Igawa T, Akahoshi M, Miyake K, Takeda A, Yoshimura A, Hamano S, Yoshida H (2008) Amelioration of human lupus-like phenotypes in MRL/lpr mice by overexpression of interleukin 27 receptor alpha (WSX-1). Ann Rheum Dis 67:1461-1467.

Szanto A, Balint BL, Nagy ZS, Barta E, Dezso B, Pap A, Szeles L, Poliska S, Oros M, Evans RM, Barak Y, Schwabe J, Nagy L (2010) STAT6 Transcription Factor Is a Facilitator of the Nuclear Receptor PPARγ-Regulated Gene Expression in Macrophages and Dendritic Cells. Immunity 33:699-712.

Taylor PR, Carugati A, Fadok VA, Cook HT, Andrews M, Carroll MC, Savill JS, Henson PM, Botto M, Walport MJ (2000) A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. J Exp Med 192:359-366.

Teh BK, Yeo JG, Chern LM, Lu J (2011) C1q regulation of dendritic cell development from monocytes with distinct cytokine production and T cell stimulation. Mol Immunol 48:1128-1138.

Telen MJ, Green AM (1989) The Inab phenotype: characterization of the membrane protein and complement regulatory defect. Blood 74:437-441.

Tenner AJ, Lesavre PH, Cooper NR (1981) Purification and radiolabeling of human C1q. J Immunol 127:648-653.

Tenner AJ, Volkin DB (1986) Complement subcomponent C1q secreted by cultured human monocytes has subunit structure identical with that of serum C1q. Biochem J 233:451-458.

Thi EP, Reiner NE (2012) Phosphatidylinositol 3-kinases and their roles in phagosome maturation. J Leukoc Biol 92:553-566.

Troegeler A, Lastrucci C, Duval C, Tanne A, Cougoule C+, Maridonneau-Parini I, Neyrolles O, Lugo-Villarino G (2014) An efficient siRNA-mediated gene silencing in primary human monocytes, dendritic cells and macrophages. Immunol Cell Biol.

Tsokos GC (2011) Systemic lupus erythematosus. N Engl J Med 365:2110-2121.

van der Touw W, Cravedi P, Kwan WH, Paz-Artal E, Merad M, Heeger PS (2013) Cutting edge: Receptors for C3a and C5a modulate stability of alloantigen-reactive induced regulatory T cells. J Immunol 190:5921-5925.

Vandivier RW, Ogden CA, Fadok VA, Hoffmann PR, Brown KK, Botto M, Walport MJ, Fisher JH, Henson PM, Greene KE (2002) Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex. J Immunol 169:3978-3986.

Veerhuis R, Nielsen HM, Tenner AJ (2011) Complement in the brain. Mol Immunol 48:1592-1603.

Villegas-Mendez A, de Souza JB, Lavelle SW, Gwyer FE, Shaw TN, van RN, Saris CJ, Hunter CA, Riley EM, Couper KN (2013) IL-27 receptor signalling restricts the formation of pathogenic, terminally differentiated Th1 cells during malaria infection by repressing IL-12 dependent signals. PLoS Pathog 9:e1003293.

Vollger LW, Uittenbogaart CH (1993) Interleukin-7 promotes the generation of phenotypically mature CD45RA positive human thymocytes in vitro. Cytokine 5:157-168.

Walport MJ, Davies KA, Botto M (1998) Clq and systemic lupus erythematosus. Immunobiology 199:265-285.

Wen Z, Xu L, Xu W, Yin Z, Gao X, Xiong S (2013) Interleukin-17 expression positively correlates with disease severity of lupus nephritis by increasing anti-double-stranded DNA antibody production in a lupus model induced by activated lymphocyte derived DNA. PLoS ONE 8:e58161.

Werfel T, Kirchhoff K, Wittmann M, Begemann G, Kapp A, Heidenreich F, Gotze O, Zwirner J (2000) Activated human T lymphocytes express a functional C3a receptor. J Immunol 165:6599-6605.

Williams KL, Lich JD, Duncan JA, Reed W, Rallabhandi P, Moore C, Kurtz S, Coffield VM, Accavitti-Loper MA, Su L, Vogel SN, Braunstein M, Ting JP (2005) The CATERPILLER protein monarch-1 is an antagonist of toll-like receptor-, tumor necrosis factor alpha-, and Mycobacterium tuberculosis-induced pro-inflammatory signals. J Biol Chem 280:39914-39924.

Wong EK, Goodship TH, Kavanagh D (2013) Complement therapy in atypical haemolytic uraemic syndrome (aHUS). Mol Immunol 56:199-212.

Xu W, Berger SP, Trouw LA, de Boer HC, Schlagwein N, Mutsaers C, Daha MR, van Kooten C (2008) Properdin binds to late apoptotic and necrotic cells independently of C3b and regulates alternative pathway complement activation. J Immunol 180:7613-7621.

Yamada M, Oritani K, Kaisho T, Ishikawa J, Yoshida H, Takahashi I, Kawamoto S, Ishida N, Ujiie H, Masaie H, Botto M, Tomiyama Y, Matsuzawa Y (2004) Complement C1q regulates LPS-induced cytokine production in bone marrow-derived dendritic cells. Eur J Immunol 34:221-230.

Yamamoto H, Fara AF, Dasgupta P, Kemper C (2013) CD46: the 'multitasker' of complement proteins. Int J Biochem Cell Biol 45:2808-2820.

Young KR, Jr., Ambrus JL, Jr., Malbran A, Fauci AS, Tenner AJ (1991) Complement subcomponent C1q stimulates Ig production by human B lymphocytes. J Immunol 146:3356-3364.

Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, Fenton MJ, Tracey KJ, Yang H (2006) HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. Shock 26:174-179.

Yuan Y, Ren J, Cao S, Zhang W, Li J (2012) Exogenous C3 protein enhances the adaptive immune response to polymicrobial sepsis through down-regulation of regulatory T cells. Int Immunopharmacol 12:271-277.

Yuan Y, Yan D, Han G, Gu G, Ren J (2013) Complement C3 depletion links to the expansion of regulatory T cells and compromises T-cell immunity in human abdominal sepsis: a prospective pilot study. J Crit Care 28:1032-1038.

Ziccardi RJ (1982) Spontaneous Activation of the First Component of Human Complement (C1) by an Intramolecular Autocatalytic Mechanism. J Immunol 128:2500-2504.

Ziccardi RJ, Cooper NR (1978) Direct demonstration and quantitation of the first complement component in human serum. Science (New York, N Y) 199:1080-1082.

Zizzo G, Hilliard BA, Monestier M, Cohen PL (2012) Efficient clearance of early apoptotic cells by human macrophages requires M2c polarization and MerTK induction. J Immunol 189:3508-3520.