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The Role of C1q in Regulation of Monocyte to Dendritic Cell

Differentiation: Implications in Autoimmunity

A Dissertation Presented

by

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Abstract of the Dissertation

The Role of C1q in Regulation of Monocyte to Dendritic Cell Differentiation:

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Growing evidence shows that the first component of complement, C1q, regulates the growth and function of cells committed to the monocyte-derived dendritic cell (DC) lineage. Because C1q regulates both innate and acquired immune responses, we postulated that C1q modulates the monocyte-DC transition at the earliest stages, i.e. at the interface between innate and acquired immunity. Our results corroborate this hypothesis and show that C1q modulates GM-CSF+IL4 induced DC differentiation, as evidenced by retention of CD14 and reduced expression of DC maturation markers and co-stimulatory molecules. C1q induced the development of at least two immature DC (iDC) subsets ($CD14^{hi}CD11c^{hi}CD16^{+/-}$). Moreover, C1q treatment resulted in significantly increased secretion of IFN-γ and MIP-1α after 24 hours, while the number of cells producing these cytokines did not notably change. C1q treatment significantly enhanced the phagocytic uptake capacity of iDCs on day 3, while it did not change their allogeneic

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immunostimulatory capacity. Taken together, these data suggest that in the absence of danger signals C1q may help maintain steady state conditions by skewing DC differentiation toward cells with monocyte-macrophage-like characteristics.

Further results revealed that freshly isolated peripheral blood monocytes carry C1q on their surface even at day 0, when they have not been exposed to DC growth factors. The binding pattern of a monoclonal antibody specific to the globular heads of C1q (gC1q) indicated that C1q is bound to monocytes and iDCs via its globular heads, presumably through gC1qR, the receptor for the globular heads of C1q. Culturing monocyte-DCs in the presence of a monoclonal antibody recognizing the C1q binding site on gC1qR resulted in the development of cells similar to those with C1q treatment, further indicating that C1q/gC1qR interaction is a requisite of early C1q signaling on these cells. Since C1q is synthesized and secreted predominantly by macrophages and DCs, we predict that a C1q-rich environment allows for specific C1q/C1q receptor interactions that may control the transition from the monocyte state (innate immunity) toward the professional antigen presenting cell state (adaptive immunity).

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Abbreviations:

Ab –   antibody

Ag –   antigen

AP –   alkaline phosphatase

APC –   antigen presenting cell

APC-conjugated –   allophycocyanin-conjugated

BrdU –   5-bromo-2-deoxyuridine

BSA –   bovine serum albumin

CR –   calreticulin

CRD –   carbohydrate recognition domain

DC –   dendritic cell

ELISA –   enzyme-linked immunosorbent assay

FBS –   fetal bovine serum

FITC –   fluorescein isothiocyanate

G4 –   GM-CSF+IL-4

GM-CSF –   granulocyte-macrophage colony-stimulating factor

HSA –   human serum albumin

iDC –   immature DC

IFN –   interferon

IgG –   immunoglobulin

IL –   interleukin

LPS –   lipopolysaccharide

mAb –   monoclonal Ab

MBL –   mannan binding lectin

M-CSF –   macrophage colony-stimulating factor

MFI –   mean fluorescent intensity

MLR –   mixed leukocyte reaction

MNC –   mononuclear cell

Mono-DC –   monocyte derived DCs

MR –   mannose receptor

OD –   optical density

pAb –   polyclonal Ab

PAMP –   pathogen associated molecular patterns

PB –   peripheral blood

PBA –   PBS containing 1% BSA and 0.01% NaN_3

PBMC –   peripheral blood mononuclear cells

PBS –   phosphate buffered saline

PE –   phycoerythrin

PMA –   phorbol 12-myristate 13-acetate

pNPP –   *p*-nitrophenyl phosphate

RA –   rheumatoid arthritis

SI –   stimulation index

SLE –   systemic lupus erythematosus

SP-A –   surfactant protein A

T_H cell –   T helper cell

TNF –   tumor necrosis factor

Acknowledgements

Before my thesis project was finalized, the first year of my research involved using RNAi to knock down gC1qR in various human cell lines. The planning and execution of this project was extensive and I could not have done it without the help of Sylvia Samaniego. From our first meeting we became great friends, and Berhane affectionately referred to us as "double trouble". Without Sylvia's help, the success of that project simply would not have been possible, and I greatly appreciate her friendship, advice and support during that time, and since then. I also must thank Ken Marcu for the warm welcome he afforded me in his lab and the generous use of his lab space during that year.

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

C1q

C1q (460 kDa) is a collagen-like, hexameric glycoprotein. It consists of similar but distinct polypeptide chains A, B and C that form six triple helices (Brodsky-Doyle et al., 1976; Reid, 1989). As a result, C1q is composed of two major structural and functional domains: six globular 'heads' linked to a collagen-like 'tail', which can be purified after the intact C1q molecule is subjected to either collagenase or peptic digestion respectively (Brodsky-Doyle et al., 1976; Knobel et al., 1975; Svehag et al., 1972) (Fig. 1). C1q associates with the Ca^{2+} -dependent $C1r_2$-$C1s_2$ tetramer (360 kDa) to form the pentameric C1, the first component of the classical complement system (Calcott and Muller-Eberhard, 1972; Lepow et al., 1963; Muller-Eberhard and Kunkel, 1961; Reid et al., 1982; Reid and Porter, 1976; Weiss et al., 1986). While most complement proteins are produced in the liver, C1q is predominantly synthesized by macrophages and dendritic cells (Bensa et al., 1983; Castellano et al., 2004a; Kaul and Loos, 2001; Schwaeble et al.,

1995; Vegh et al., 2003). C1q circulates in plasma at a concentration of 70-160 μg/ml (Dillon et al., 2009; Hughes-Jones, 1977; Schuller and Helary, 1983), but like other components of innate immunity, it is produced in higher concentrations at inflammatory sites (Breitner et al., 1995; Soda et al., 1988). Approximately 80% of C1q contained in the plasma is associated with the C1 complex, while the remaining portion is in its monomeric, 'free' form (Sjoholm et al., 1985). Out of all proteins found in the serum C1q is the most positively charged (Heinz, 1989), which allows it to interact through ionic bonds with a multitude of negatively charged molecules (Hughes-Jones and Gardner, 1978).

C1q belongs to the collectin (**col**lagen containing **lectin**) family of molecules, which contain collagen-like sequences contiguous with non-collagen-like stretches. Members of this family include: mannan binding lectin (MBL), lung surfactant protein A (SP-A), collectin-43 and conglutinin, which can bind to the C1q receptor cC1qR via their collagen-like regions (Malhotra et al., 1992; Malhotra et al., 1990a; Reid, 1989). Most collectins differ from C1q in that they contain carbohydrate recognition domains (CRDs) that recognize glycoconjugates containing mannose and fucose on microorganisms but not on self-proteins (Hoppe and Reid, 1994; Lu et al., 1993). C1q, on the other hand, contains collagen sequences which allow it to bind to protein motifs in immunoglobulin (Ig)G or IgM (Lu et al., 1993). Thus C1q can bind to immune complexes and engage in complement-mediated microbial killing and phagocytosis (Bobak et al., 1987; Leist-Welsh and Bjornson, 1982). As a result, C1q deficiency is associated with increased

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Figure 1. Schematic model of the structure of C1q.

C1q consists of 18 polypeptide chains of 3 different types (A, B, C). The three chains form a triple helix and there are six triple helices in the molecule. The C-terminal ends of each chain in the triple helix merge to form a globular head; and there are six 'heads' in the molecule.

flexible
hinge

collagen-like
'tail'
(cC1q)

globular
'heads'
(gC1q)

A B C

N-term

C-term
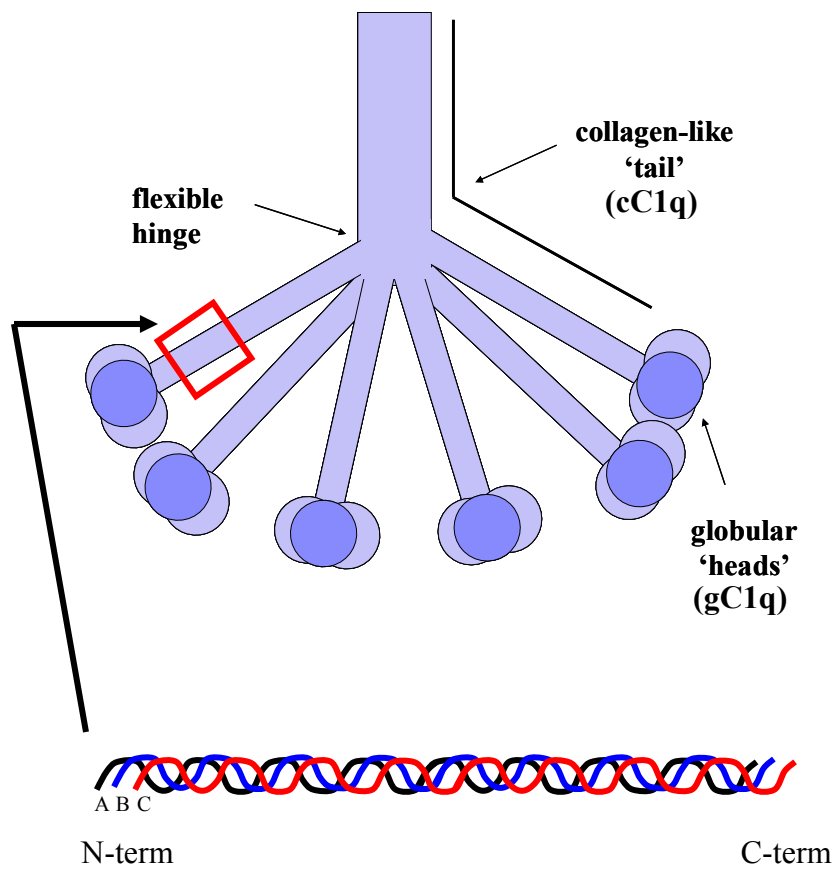
susceptibility to microbial infections including otitis media, meningitis, and pneumonia (Kuis et al., 1988; Pickering et al., 2008; Prellner et al., 1989; Vassallo et al., 2007).

Over the past 20 years, convincing evidence has been accumulated showing that C1q and other members of the collectin family play a role in recognition and removal of altered self and apoptotic cells (Korb and Ahearn, 1997; Ogden et al., 2001; Vandivier et al., 2002). The mechanism of this removal involves the recognition of the apoptotic cell surface and initiation of phagocytic uptake by macrophages and DCs through interaction with C1q receptors expressed both on the phagocytic cell, (e.g. cC1qR/CD91) (Ogden et al., 2001; Vandivier et al., 2002) and the apoptotic cell (gC1qR and phosphatidylserine) (Paidassi et al., 2008). Defective clearance of immune complexes and apoptotic cells, in turn, may lead to immune recognition of normally hidden epitopes – a critical immunopathogenic event leading to autoimmune disease.

C1q receptors

C1q receptors mediate many immunologic functions including phagocytosis and clearance of apoptotic cells. There are at least two types of distinct, ubiquitously expressed cell surface molecules which bind human C1q: gC1qR, the receptor for the globular heads, and cC1qR, the receptor for the collagen tail (Chen et al., 1994; Feng et al., 2002; Herwald et al., 1996; Ogden et al., 2001; Peerschke et al., 1994; Sim et al., 1998; Vegh et al., 2006). Both of these receptors are multiligand binding proteins capable of inducing a wide range of cellular functions involved in innate and adaptive immunity.

cC1qR

The cC1qR molecule (60 kDa) is a highly conserved, acidic glycoprotein, which binds C1q via its collagen-like 'tail' (Ghebrehiwet, 1986). It is a homologue of the extracellular ecto-calreticulin (CR) and is therefore sometimes referred to as cC1qR/CR or 'collagen receptor' (Malhotra, 1993; Malhotra et al., 1990b). In addition to its calcium binding properties, cC1qR/CR has been described as a molecular chaperone (Nauseef et al., 1995; Wada et al., 1995), an extracellular compartment protein (Somogyi et al., 2003), an intracellular mediator of integrin function (Dedhar, 1994; Feng et al., 2002), an inhibitor of steroid hormone-regulated gene expression (Burns et al., 1994; Dedhar et al., 1994; Platet et al., 2000), and a receptor for other collectins (Coppolino and Dedhar, 1998; Malhotra, 1993).

Since cC1qR does not contain a transmembrane domain or a GPI-anchor attachment site, it needs to forge docking-signaling partnerships with adaptor molecules for signal transduction. One such molecule is CD91 (Basu et al., 2001), which was shown to participate in the engagement of cell surface cC1qR by C1q on monocytes to initiate macropinocytosis and uptake of apoptotic cells (Ogden et al., 2001; Vandivier et al., 2002). However, antibody blockade of either CD91 or calreticulin did not completely inhibit the uptake process (Vandivier et al., 2002). Furthermore, recent studies using macrophages derived from CD91-/- mice showed that CD91 is not required for the C1q-

triggered enhancement of phagocytosis (Lillis et al., 2008), suggesting the existence of alternative receptor components that may participate in C1q-mediated phagocytosis. Alternative molecules that have been identified as co-receptors of cC1qR include scavenger receptor A on antigen presenting cells (Berwin et al., 2003), CD59 on neutrophils (Ghiran et al., 2003), $\alpha2\beta1$ integrin and glycoprotein VI on resting platelets (Elton et al., 2002), MHC class I on T cells (Santos et al., 2004), and CD69 on human peripheral blood mononuclear cells (PBMCs) (Vance et al., 2005).

Some studies show, that while C1q can bind stably to cC1qR, when both molecules are present in equal quantities in their natural conformation, no interaction takes place (Steino et al., 2004). However, immobilized, heat-treated, or IgG-bound C1q shows a strong, rapid and specific binding to cC1qR, suggesting that cC1qR is a receptor for an altered conformation of C1q (Steino et al., 2004). Such altered conformational states are induced by binding of C1q to IgG oligomers (Vandenberg and Easterbrook-Smith, 1986) and immune complexes (Heinz, 1989). These data indicate that soluble, monomeric C1q may not be able to bind to cC1qR until it undergoes a conformational change in response to binding to IgG, immune complexes, pathogenic surface materials or other structures via its globular 'head' regions. Additionally, when C1q is associated with the $C1r_2$-$C1s_2$ tetramer within the C1 complex, only the globular 'heads' of C1q are available for binding. After removal of the $C1r_2$-$C1s_2$ complex by C1-inhibitor, the collagen-like 'tail' of C1q becomes free to bind to cell-surface cC1qR (Reid, 1989).

gC1qR

Another well-described C1q receptor, gC1qR (p32/p33/HABP1), binds to the globular 'heads' with high affinity (K_d of 13.5 nM ($k_{on} = 5 \times 10^4$ $M^{-1}s^{-1}$; $k_{off} = 6.8 \times 10^{-4}$ s^{-1}) under physiologic conditions (Ghebrehiwet et al., 1994; Herwald et al., 1996; Peerschke et al., 1994). It is a highly negatively charged homotrimer, comprised of three 33-kDa chains with a ubiquitous and multi-compartmental distribution including on the cell surface. The molecule is synthesized as a 282 aa pre-pro-protein, which is initially targeted for the mitochondria (Dedio et al., 1999; Ghebrehiwet et al., 1994; Jiang et al., 1999; van Leeuwen and O'Hare, 2001). Mature gC1qR is generated by removal of the first 73 residues during post-translational processing (Ghebrehiwet et al., 1994; van Leeuwen and O'Hare, 2001).

The presence of gC1qR was confirmed on the surface of human macrophages and monocyte-derived DCs, in addition to many other cell types (Chen et al., 1994; Leigh et al., 1998; Peerschke et al., 1994; Peerschke et al., 1996; Vegh et al., 2003; Waggoner et al., 2005). The highly asymmetric surface charge distribution of gC1qR, with one face containing significantly higher negatively charged residues (solution or S-Phase) than the opposite surface (membrane or M-Phase), indicates that the two faces carry out different functions (Ghebrehiwet et al., 2002).

Due to the ability of gC1qR to recognize and bind to a diversity of microbial, cellular and plasma derived ligands, many pathogens employ immune escape mechanisms to exploit the normal regulatory functions of C1q/gC1qR. Microbes that interact with and exploit gC1qR include HIV (Berro et al., 2006), adenovirus (Matthews and Russell, 1998; Ohrmalm and Akusjarvi, 2006), Epstein-Barr virus (Wang et al., 1997), Herpesvirus Saimiri (Hall et al., 2002), rubella virus (Beatch et al., 2005; Beatch and Hobman, 2000; Mohan et al., 2002), hepatitis B virus (Laine et al., 2003), hepatitis C virus (HCV) (Kittlesen et al., 2000; Yao et al., 2003), *L. monocytogenes* (Braun et al., 2000), *S. aureus* (Nguyen et al., 2000; Peerschke et al., 2006), and *B. cereus* (Ghebrehiwet et al., 2007). The fact that so many types of microbial pathogens possess strong affinity for gC1qR further implies that gC1qR plays an important role in microbial pathogenesis as well as immune regulation. For example, in vitro studies have shown that HCV, which binds gC1qR via the same or overlapping binding site as C1q, employs gC1qR on monocyte-DC precursors to prevent DC immunogenic activity (Waggoner et al., 2005). HCV core protein also binds to gC1qR on T cells resulting in reduced T cell responses (Kittlesen et al., 2000; Yao et al., 2001a; Yao et al., 2001b). Furthermore, DCs isolated from patients infected with HCV lack the capacity to stimulate T cells in a mixed leukocyte reaction (MLR) (Kanto et al., 1999). Taken together, these observations suggest that the normal physiologic function of gC1qR include prevention of DC stimulation and reduced T cell activation.

The capacity of gC1qR to elicit a diverse array of biologic responses and transduce intracellular signals has been shown by several investigators using a variety of cell types (Feng et al., 2002; Kittlesen et al., 2000; Meenakshi et al., 2003; Moorman et al., 2005; Peerschke et al., 1994; Peerschke et al., 1996; Waggoner et al., 2005; Yao et al., 2003; Yao et al., 2004; Yao et al., 2001a; Yao et al., 2005), but information on how gC1qR regulates human macrophage and dendritic cell activity is still lacking. One study shows that signaling through gC1qR inhibits TLR4-induced IL-12 production without increased IL-10 or TGF-β on macrophages and dendritic cells. Activation of PI3K and subsequent phosphorylation of Akt was also observed following gC1qR cross-linking (Waggoner et al., 2005). These data indicate that gC1qR is involved in the regulation of immunosuppressive signaling mechanisms in these cells. However, like cC1qR, it lacks a transmembrane segment, a deficiency that is overcome by formation of docking/signaling partnerships with transmembrane proteins. Putative docking/signaling partners that have been indicated in association with gC1qR are β1-integrins on endothelial cells (Feng et al., 2002), vasopressin V2 receptor (Bellot et al., 2009), and alpha(1B)-adrenergic receptor on the COS 7 cell line (Xu et al., 1999).

C1q and Autoimmunity

Aberrations in C1q structure and/or function have been linked to various disease processes. The clinical association between C1q and autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) is well established. In RA, antibodies to the collagen-like subunit on the A-chain of C1q may cross-react with collagen type II and contribute to the disease process that leads to tissue destruction and inflammation (Maeurer et al., 1993; Trinder et al., 1997). A synthetic decapeptide corresponding to the A-chain of C1q injected into DBA/1 mice delays the onset and reduces the severity of collagen-induced arthritis (Maeurer et al., 1992; Maeurer et al., 1993). Possibly during the inflammatory process the conformation of C1q may be altered, and result in the generation of autoantibodies. In fact, autoantibodies to C1q impair C1q function in animal models of RA indicating a regulatory role for C1q in suppressing immune activity, and further suggesting that C1q may be a link between the early inflammatory response in the RA joint and the cartilage destruction that occurs later in the disease (Trinder et al., 1996; Trinder et al., 1997).

Deficiency of C1q is considered to be a strong susceptibility factor for SLE (Cortes-Hernandez et al., 2004; Fremeaux-Bacchi et al., 1996; Ghebrehiwet and Peerschke, 2004a; Steinsson et al., 1983; Walport et al., 1998). Out of the known human cases of homozygous C1q deficiency, the vast majority ($\geq$95%) have developed clinical syndromes closely related to SLE, with rashes, glomerulonephritis, and central nervous

system disease (Walport, 2002; Walport et al., 1998). In addition, 33% of SLE patients have high affinity autoantibodies to C1q, which are mostly directed to a neo-epitope expressed in the A-chain of C1q (Walport, 2002; Walport et al., 1998). Although most studies suggest that it is the failure to properly clear apoptotic cells in the absence of C1q that results in these cells becoming immunogenic (Hurst et al., 1984; Walport et al., 1998), recent observations challenge this idea. Disruption of other apoptotic uptake processes, such as those mediated by CD14 (Devitt et al., 2004), β3 or β5 integrin (Lucas et al., 2006), and mannose-binding lectin (Stuart et al., 2005), all result in accumulation of apoptotic bodies without triggering autoimmunity. In recent years mounting evidence has emerged showing that aside from the recognition and triggering of the classical complement pathway, C1q is also a powerful modulator of the acquired immune response. Based on these observations we propose that the absence or defective expression of C1q/C1qR could lead to a loss of peripheral tolerance as a cumulative result of impaired apoptotic cell clearance in conjunction with faulty control of C1q mediated adaptive responses.

The Complement System and Adaptive Immunity

Although the primary function of the complement system is to recognize and destroy pathogenic microorganisms, multiple immunologic functions are mediated by its various components and activation products. Complement components and/or their fragments have been shown to play an important role in antigen-specific immune responses, including antigen processing or presentation, T cell proliferation and differentiation, B-cell activation, and the maintenance of tolerance (Sohn et al., 2003). Opsonization of apoptotic cells by autologous iC3b has been shown to facilitate clearance by immature DCs (iDCs). This event also altered DC maturation, as evidenced by downregulation of the T cell co-stimulatory CD86 and the MHC class II receptor HLA-DR and enhanced expression of the homing receptor CCR-7 (Verbovetski et al., 2002). In addition, the binding of iC3b to DCs has been shown to downregulate the release of proinflammatory cytokines including TNF-α, IL-1β, and IL-12p70 (Morelli et al., 2003). Although the central role played by C3 and its activation fragments in antigen-specific immune responses is well documented, and its role in DC biology has recently become the central theme of many laboratories, the role of C1q in immune regulation is rapidly gaining importance. Like C3, C1q participates in phagocytosis (Nauta et al., 2004; Ogden et al., 2001; Taylor et al., 2000; Vandivier et al., 2002), chemotaxis (Kuna et al., 1996; Vegh et al., 2006), and regulation of B and T cell responses (Chen et al., 1994; Ghebrehiwet et al.,

1990; Habicht et al., 1987). Additionally, in recent years there has been a growing recognition concerning the role of C1q in regulating DC activity, including its effects on DC differentiation and function.

Dendritic Cells

Dendritic cells (DCs) are instrumental in modulating adaptive immunity while they are also specialized in detecting and responding to signals from the microenvironment. Because of the strong association between autoimmunity and complement deficiencies, interaction between complement and DCs is of fundamental importance. Dendritic cells are a complex lineage of antigen presenting cells (APCs) that orchestrate a variety of immune responses. Although B and T cells mediate acquired immunity, their function is under the control of DCs (Romani et al., 1989; Steinman, 2007; Young and Steinman, 1990). DCs in various stages of maturity capture, process and present antigens, express lymphocyte co-stimulatory molecules when activated, migrate to lymphoid organs, and secrete cytokines to initiate immune response. T lymphocytes require the Ag to be processed and presented to them via MHC molecules on the surface of DCs and other APCs (Banchereau et al., 2000; Banchereau and Steinman, 1998; Buus and Werdelin, 1986; Romani et al., 1989). Two types of peptide-binding molecules are found on APCs: MHC class I (MHC I), which stimulates cytotoxic CD8+ T cells and MHC class II (MHC II), which stimulates helper CD4+ T cells. In addition to their ability to activate lymphocytes, DCs can also tolerize T cells to self-antigens by a variety of mechanisms including the production of regulatory cytokines such as IL-10 and the induction of regulatory T cells (Albert et al., 1998; Banchereau and Steinman, 1998; Figdor et al.,

2002; Inaba et al., 1997; Redmond et al., 2009; Steinman et al., 2003; Steinman et al., 2000).

DC precursors circulate in the bloodstream as monocytes, which are continuously generated from bone marrow progenitors (Egner et al., 1993; Romani et al., 1994; Shortman and Caux, 1997; Thomas et al., 1993; Thomas and Lipsky, 1996; Young and Steinman, 1996). Migration into non-lymphoid organs induces differentiation of DC precursors into iDCs that become resident tissue cells of the interstitium of peripheral organs or skin (Breathnach and Katz, 1984; Kupiec-Weglinski et al., 1988; Santiago-Schwarz, 1999; Steinman, 2007). These tissue-resident DCs are thought to be in an immature state and are specifically characterized by high phagocytic activity and the ability to capture self and foreign antigens through specific lectins (e.g. DC-SIGN, Langerin, mannose receptors) and other surface molecules (e.g. $\alpha_v\beta_5$ integrin, CD91) (Albert et al., 1998; Sallusto et al., 1995). Therefore, DCs can interact with virtually every antigen present in the periphery, which are then engulfed, processed and presented as peptide-MHC complexes on the DC surface (Inaba et al., 1997).

Following uptake of self antigens, tolerogenic mechanisms exist to prevent inappropriate autoimmune responses. Under steady-state conditions DCs remain immature and start migrating towards lymph nodes upon partial activation signaling (Sauter et al., 2000; Williams et al., 2008). Once they have reached the T cell area, these semi-mature DCs may induce tolerance by numerous suggested mechanisms (Sauter et al., 2000; Steinman et al., 2000; Williams et al., 2008). In contrast, when DCs encounter

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a peripheral microenvironment characterized by pro-inflammatory factors and antigenic material such as LPS (Suri and Austyn, 1998), bacterial DNA (Schattenberg et al., 2000) and double stranded RNA (Cella et al., 1999), migration toward lymphoid organs and terminal maturation is triggered by these molecules and their recognition by specific Toll-like receptors (Kadowaki et al., 2001). Cytokines such as TNF-α and IL-1β found in the inflammatory compartment are also important participants in the DC maturation/activation process (Jonuleit et al., 1997). The maturation of DCs is marked by up-regulation of co-stimulatory molecules (CD40, CD80, CD86, CD58), secretion of cytokines (TNF-α, IL-6, IL-12p70), the loss of endocytic/phagocytic receptors, high levels of MHC I, II, CD83, and acquisition of high cellular mobility (Banchereau et al., 2000; Inaba et al., 1997; Young and Steinman, 1996). Mature DCs migrate to the T cell areas of local lymph nodes, where priming of naïve CD4+ T cells takes place through engagement of the appropriate receptors and CD40L (Green and Jotte, 1985; Langhoff and Steinman, 1989; Romani et al., 1989). The type of T cell subsets activated by DCs is dependent on several factors including the DC subset involved, the nature and dose of the antigen, and the cytokines present in the environment where the DC-microorganism interaction occurs.

Distinct subsets of DC precursor populations have been described to arise from PBMCs treated with GM-CSF+IL-4. Included in these populations are the major subset of CD14$^-$CD11c$^+$ cells, and minor subsets, such as CD14$^-$CD11c$^-$ and CD16$^{+/-}$ CD14$^+$CD11c$^+$ (Grage-Griebenow et al., 2001a; MacDonald et al., 2002). Phenotypic

diversity among DC subsets is thought to reflect a functional dissociation, imparting them with different immunoregulatory capabilities (Grage-Griebenow et al., 2001a). While the majority of iDCs (CD14$^-$CD11c$^+$) actively ingest potentially antigenic material as a prerequisite for an immunogenic response; failure to undergo terminal maturation into APC results in a potentially tolerogenic state (Banchereau et al., 2000; Castellano et al., 2004b; Grage-Griebenow et al., 2001a; MacDonald et al., 2002; Shortman and Caux, 1997; Steinman et al., 2003; Steinman et al., 2000). Intriguingly, a population of CD14^{hi}CD16$^+$ iDCs has recently been described to exhibit characteristics of both monocytes and DCs, possibly enabling these cells to activate innate, as well as adaptive immune responses (Grage-Griebenow et al., 2001a; Grage-Griebenow et al., 2001b). Aside from expressing accessory molecules associated with T cell stimulation, retention of CD14 on these cells indicates signaling potential via monocyte- and macrophage-associated receptors.

Immature DCs, but not mature DCs, are a primary source of active C1q (Cao et al., 2003; Castellano et al., 2004a; Schwaeble et al., 1995; Vegh et al., 2003). Indications that C1q may function as an autocrine signal in DCs and regulate both innate and adaptive properties have emerged in the past few years. Human monocyte-derived iDCs express high levels of both C1q receptors, gC1qR and cC1qR, and their surface levels decrease in a maturation-dependent manner, suggesting specific roles for C1qRs in iDC function (Vegh et al., 2003).

C1q and Dendritic Cells

The role of C1q in the regulation of DC differentiation and function has been much studied and debated in recent years. Most of the work has focused on determining the potential regulatory role of C1q during DC maturation, after the commitment to the DC lineage has already taken place. These experiments show that LPS treated human iDCs that grew in the presence of C1q showed decreased CD80, CD83 and CD86 expression as compared to non-treated iDCs. The cells also produced significantly less IL-6, TNF-α and IL-10 and showed decreased allostimulation of T helper (T_H) 1 cells in a mixed leukocyte reaction (MLR) (Castellano et al., 2007b). The results suggest that C1q treated iDCs may be resistant to LPS-induced maturation. Using murine bone marrow-derived dendritic cells, Yamada and colleagues (Yamada et al., 2004) showed that C1q treatment suppressed IL-12p40 production, reduced the activity of NF-κB and delayed the phosphorylation of p38, c-Jun N-terminal kinase, and extracellular signal-regulated kinase after LPS-stimulation or CpG oligodeoxynucleotide induction. These data further indicate that C1q may function by suppressing proinflammatory responses after DC activation.

However, in apparent conflict with the two previous studies, C1q deficient DCs from mice and humans showed decreased production of IL-12p70 after treatment with anti-CD40 antibodies. Additionally, the murine cells also had impaired expression of CD80 and CD86, decreased phosphorylation of p38 and ERK1/2, and showed a reduced

ability to induce T_H-1 differentiation (Baruah et al., 2009). These experiments imply that C1q sustains DC maturation by supporting the expression of co-stimulatory molecules and positively regulating T cell stimulatory capabilities of DCs.

In order to imitate the role of C1q as an opsonin in vitro, some studies employed immobilized C1q. Nauta and her colleagues found that the uptake of C1q-opsonized apoptotic cells by iDCs stimulated the production of IL-6, IL-10, and TNF-α, without an effect on IL-12p70 (Nauta et al., 2004). Additionally, iDCs placed on immobilized C1q, gC1q or cC1q showed enhanced maturation, translocation of NF-κB to the nucleus and enhanced secretion of IL-12 and TNF-α, in addition to elevated T_H1-stimulating capacity (Csomor et al., 2007). Taken together, these studies suggest that "fixation" of C1q may support DC maturation, while soluble C1q might decrease a pro-inflammatory response.

So far, very little data is available on how soluble C1q that is present in the plasma and interstitial tissues under steady state conditions might regulate DC differentiation during the earliest stages of mono-DC growth. Understanding the molecular mechanisms of how C1q regulates adaptive immune functions via iDCs in the absence of infection or inflammation is therefore highly significant. Studies from our laboratory and others have shown that C1q acts as a chemoattractant to iDCs, but not mature DCs. C1q induced migration is mediated through ligation of both gC1qR and cC1qR and activation of Akt and MAPK pathways (Liu et al., 2008; Vegh et al., 2006). C1q treatment during DC differentiation was also shown to give rise to CD1a^{+}DC-SIGN^{+} iDCs with high phagocytic capacity, and low expression of CD80, CD83 and CD86

(Castellano et al., 2007b). Because this narrow window of differentiation represents the important interface between innate and adaptive immunity, more work is needed to explore this crucial stage.

Significance

While there is agreement that the expression of both C1q and C1qRs is decreased upon terminal DC maturation, the ambiguous role of C1q is reflected in conflicting reports of either inflammatory induction (Csomor et al., 2007; Gardai et al., 2003), or suppression (Castellano et al., 2007a) of monocytes and DCs. These contradictory findings may be due to differences in the maturation stage of the DCs studied, the time, duration and concentration of C1q treatment, or the effects of endotoxin present in the assay system. Importantly, there is little or no information related to the expression and regulatory role of C1q and C1qRs during the earlier stages of DC development which reflect optimal antigen uptake/processing activity. The data presented here suggest a novel role for C1q at the level of monocyte-to-DC differentiation. The evidence in support of this conclusion is that monomeric, soluble C1q that is free of antigenic 'cargo' may skew DC differentiation to promote the development of a 'hybrid' cell type that has both innate and acquired characteristics. Thus, while C1q may provide active protection from autoimmunity by silencing or regulating autoreactive immune cells, its absence or defective expression could lead to cellular activation as a cumulative result of impaired apoptotic cell clearance in conjunction with negative signaling. Our findings therefore provide novel and important functions for C1q and indicate that a C1q/C1qR system specifically targets DCs as they transition from the monocyte state (innate immunity)

toward the professional APC state (adaptive immunity). In the long term, our data should

provide new groundwork for future studies related to innate and acquired immunity.

<u>Innovation</u>

It has long been suspected that deficiency in the early components of the classical pathway of complement play a role in the etiology and pathogenesis of autoimmune diseases such as SLE. The correlation between C1q deficiency and SLE is particularly striking as it can fairly accurately predict that individuals with C1q deficiency will develop SLE. What has been unknown is the precise definition of the molecular underpinnings by which C1q triggers or exacerbates the disease. In our attempt to address this fundamental question, we have uncovered a *novel* immunological role for C1q in innate and adaptive immunity: that of a molecular switch that dictates the monocyte-to-DC transition. The data presented here are novel with the potential to have a significant impact in the area of autoimmune disease in general and SLE in particular.

CHAPTER 2

MATERIALS AND METHODS

Chemicals and Reagents

The following reagents and chemicals were purchased or obtained from the sources indicated: Lymphoprep (Axis-Shield, Oslo, Norway); Annexin V-FITC, Annexin V Binding Buffer, GolgiPlug, FACS/Lyse solution (Becton-Dickinson (BD), Mountain View, CA); cell lysis buffer (Cell Signaling Technology, Danvers, MA); C1q (CompTech, Tyler, TX); heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT); 100x Penicillin/Streptomycin, RPMI 1640 (Gibco-Invitrogen, Grand Island, NY); human serum albumin (HSA) (Immuno-US, Rochester, MI); Fix/Perm kit, fluorescein conjugated transferrin (Invitrogen, Carlsbad, CA); human recombinant (r) granulocyte-macrophage colony-stimulating factor (GM-CSF), human rIL-4, human r macrophage colony-stimulating factor (M-CSF), human r interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) (Peprotech, Rocky Hill, NJ); Detoxi-Gel Endotoxin Removing Gel, *p*-nitrophenyl phosphate (pNPP), EZ-Link Sulfo-NHS-LC-Biotin, Protein A agarose, F(ab')$_2$ Micro Preparation Kit (Pierce, Rockford, IL); human IgG, formalin, ExcelArray

Human Inflammation I array kit, fluorescein-conjugated dextran, propidium iodide, lucifer yellow, phorbol 12-myristate 13-acetate (PMA), ionomycin (Sigma-Aldrich, St. Louis, MO); BrdU (5-bromo-2-deoxyuridine) ELISA kit (Roche; Indianapolis, IN); and Immu-Mount (Thermo Fisher, Waltham, MA).

Antibodies used were against: CD14 and CD83 (Biolegend, San Diego, CA); CD16, HLA-DR, CD86, CD11c, IFN-γ, TNF-α, MIP-1α, MIP-1β, IL-10, IL-12, IL-4 (BD); cC1qR (Serotec, Raleigh, NC); monoclonal (mAb) and polyclonal antibody (pAb) against C1q (Quidel, Santa Clara, CA); fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG F(ab')$_2$ or sheep anti-rabbit IgG F(ab')$_2$ (Invitrogen); and alkaline phosphatase (AP)–conjugated rabbit anti-goat IgG (Pierce).

Because of the sensitivity of DCs to endotoxin and contaminating pathogen associated molecular patterns (PAMPs), highly purified and endotoxin-poor reagents and proteins were purchased when possible. In addition, special efforts were made to ensure that proteins such as C1q and antibodies used in the cell culture were low in endotoxin by passage over Detoxi-Gel columns using pyrogen-free buffers. The endotoxin removal efficiency in one passage is ≥ 99% (Pierce).

Monoclonal and polyclonal antibodies to gC1qR, C1s, C1r, C1 inhibitor and factor B

The production, characterization and purification of various monoclonal and polyclonal antibodies to the gC1qR protein, as well as to various peptides derived from the molecule have been previously described (Ghebrehiwet et al., 1996). Similarly, pAbs to C1s, C1r, C1 inhibitor and factor B were prepared and constitute part of the antibody bank in our laboratory. F(ab')$_2$ IgG fragments were prepared using a Pierce kit according to the manufacturer's instructions.

Generation of monocyte-derived DC

Mononuclear cells (MNCs) were isolated from heparinized whole blood of apparently healthy human donors using Lymphoprep density gradient centrifugation. The cells were then cultured at a concentration of 10^6 cells/ml in RPMI 1640 containing 10% heat inactivated FBS, 100 U/ml penicillin/streptomycin, supplemented with 50 U/ml rGM-CSF and 50 ng/ml rIL-4 in Teflon vials to generate monocyte-DCs. As previously reported (Santiago-Schwarz et al., 1996), this suspension culture system coupled with flow cytometric analysis, allows accurate assessment of the effects of the various stimuli

on developing DC, monocyte, and lymphoid subpopulations. After purification of mononuclear cells using Lymphoprep, monocyte/DCs comprised 15-20% of the culture system. In addition to monocyte/DCs, lymphocytes were present, while all other contaminating cell constituents (platelets, red blood cells, polymorphonuclear cells) were absent from the culture, as assessed by scatter profiles and Wright stain analysis. Monocyte/DC subset gates were calculated on the basis of forward and side light scatter profiles and expression patterns of myeloid cell/DC associated markers (HLA-DR, CD86, CD83, CD14, CD16, and CD11c). Lymphocytes were characterized by distinct light scatter patterns and lack of myeloid/DC markers. Alternatively, when indicated, monocytes were further purified by adherence selection on polystyrene plates. In these experiments the final cell concentration after purification was 0.5×10^6 cells/ml. Highly purified C1q at a concentration of 25 µg/ml or as indicated in the experimental procedure was added to the culture starting from day 0. For experiments utilizing F(ab)'2 anti-gC1qR specific to the C1q binding site (60.11), purified 60.11 F(ab)'2 were added to the culture at 30 µg/ml on day 0. The cells were harvested every day for flow cytometric analysis. All cell culture conditions and reagents were low in endotoxin and viability of cells was usually ≥95%. Finally, each experiment performed using the suspension cell system was repeated with purified adherent monocytes (n=2-3) yielding similar results (data not included).

Flow cytometry assisted analysis of cell surface markers

Cells were removed from the culture daily and washed twice in PBA staining buffer (PBS containing 1% BSA and 0.01% NaN_3). Non-specific binding was blocked by incubating the cells with 1 mg/ml human IgG in 100 μl PBA / $1x10^6$ cells (30 min, 4°C) and primary Abs conjugated to FITC, phycoerythrin (PE) or allophycocyanin (APC) fluorochromes or the appropriate isotype-matched controls were then added to the cells (30 min, 4°C). For unconjugated primary Abs, the cells were first incubated with the primary Abs or their isotype-matched controls, then washed twice in PBA buffer and further incubated with FITC conjugated goat anti-mouse IgG F(ab')2 or sheep anti-rabbit IgG F(ab')2 (30 min, 4°C). The cells were then washed twice in cold PBA, fixed in 1% formalin and assessed by flow cytometric analysis using FACSCalibur (Becton-Dickinson, Mountain View, CA). For each analysis, 10,000 events were collected and the data obtained was analyzed using CellQuest Pro software (BD).

Multiparametric analysis of apoptosis

Cells were removed from the culture daily, washed twice in cation-free low endotoxin PBS, resuspended in RPMI 1640 containing 1% human serum albumin (1% HSA/RPMI), and incubated with fluorochrome labeled antibodies or isotype controls (20 min, 4°C).

After labeling, cells were washed twice in 1% HSA/RPMI, resuspended in 100 µl 1% HSA/RPMI, followed by 400 µl of 1x Annexin V Binding Buffer, and incubated with Annexin V-FITC (20 min, 4°C). The cells were finally fixed in 1% PBS buffered formalin and acquired by flow cytometric analysis as described above. Apoptotic events were also assessed by morphological analysis of Wright stained cells using light microscopy.

Antigen uptake

Mononuclear cells were isolated as previously indicated; monocytes were further separated by plastic adherence (2 h, 37°C) and cultured in DC growth factors ±C1q (25 µg/ml) for 3 days. On day three the cells were washed twice in PBS and resuspended in 10% FBS/RPMI ($1x10^6$ cells/ml). The cells were then incubated with FITC-dextran (1 mg/mL), Lucifer yellow (250 µg/ml) or FITC-transferrin (250 µg/ml) (30 mins, 37°C or 4°C). The incubation was stopped by washing the cells three times with ice-cold PBA. After fixing the cells with 1% formaldehyde, phagocytic uptake was analyzed using a FACSCalibur device (Becton Dickinson).

Measurement of total intracellular cytokine accumulation by flow cytometry

Monocytes were enriched from MNC preparations by plastic adherence and cultured for 24 hrs in 10% FBS/RPMI at a concentration of 0.5×10^6/ml supplemented with GM-CSF + IL-4 ± 25 μg/ml C1q. GolgiPlug, a protein transport inhibitor, was added to the cultures at a concentration of 1 μg/ml for the last 18 hrs of culture. Cells were than harvested and washed twice in PBA. Non-specific binding was blocked by incubating the cells with 1 mg/ml human IgG in 100 μl PBA / 1×10^6 cells (30 min, 4°C), followed by permeabilization using a commercially available permeabilizing agent (Fix/Perm kit) according to the manufacturer's instructions. Primary Abs conjugated to FITC, PE or APC fluorochromes or the appropriate isotype-matched controls were then added to the cells (20 min, room temp.). The cells were washed twice in cold PBA, fixed in 1% PBS buffered formalin and assessed by flow cytometric analysis using FACSCalibur (Becton-Dickinson, Mountain View, CA). Region analysis was set according to forward- and side-scatter patterns and expression patterns of myeloid DC associated markers (HLA-DR, CD86, CD14, and CD11c); positive values were determined according to isotype controls. For each analysis, 5,000 monocyte events were collected and the data obtained were analyzed using CellQuest Pro software (BD).

Determination of cytokine secretion by cytokine array

Monocytes were enriched from MNC preparations by plastic adherence and cultured at a concentration of 0.5 x 10^6/ml in 10% FBS/RPMI supplemented with GM-CSF + IL-4 +/- 25 μg/ml C1q (24 hrs, 37°C). Cell culture supernatants were collected after 24 hrs and C1q was removed from the supernatants by incubating with equal volume of Protein A agarose while rocking (overnight, 4°C) to prevent unwanted background signal due to C1q binding to the Fc region of the Ab used. Cytokine analysis was performed using a multiplex cytokine array kit by determining fluorescence intensity and relative cytokine concentrations according to the manufacturer's instructions.

Determination of the effect of IFN-γ and/or TNF-α on DC differentiation

Monocytes were enriched from MNC preparations by plastic adherence and cultured at a concentration of 0.5 x 10^6/ml in 10% FBS/RPMI supplemented with GM-CSF + IL-4 in addition to 25 μg/ml C1q, 200U/ml IFN-γ, 50 U/ml TNF-α, or IFN-γ + TNF-α for 3 days. The cells were then harvested and the expression of DC and monocyte specific cell surface markers was assessed by flow cytometry as explained above.

Allogeneic mixed leukocyte reaction (MLR)

Monocytes were enriched from MNC preparations by plastic adherence for two hours. Stimulator cells were generated for the MLR by culturing monocytes in 10% FBS/RPMI at a concentration of 0.5×10^6/ml in the presence of GM-CSF+IL-4 ± 25 μg/ml C1q for 3 days. M-CSF treated monocyte-macrophages were used as non-APC control stimulators and 48 hr LPS treatment was used in addition to the DC growth factors to generate positive control stimulators. On day 3 stimulator cells were removed from cultures, centrifuged in RPMI and adjusted to equal concentrations in 10% FBS/RPMI. Allogeneic responder T cells were purified using a Nylon wool column from PBMCs of healthy individuals. Stimulator cells were added to 96-well U-bottom microtiter plates, and mixed with responder cells in 1:2, 1:4, 1:8 (stimulator : responder) ratio/well. Control cultures containing stimulator or responder cells alone were used as non-proliferating controls. Proliferation was measured on days 6–7 by a commercially available colorimetric BrdU ELISA assay according to the manufacturer's instructions. Stimulation index (SI) was calculated as (BrdU labeling index of mixed cells) / (BrdU labeling index of responder cells alone).

Measurement of T_H1/T_H2 responses

MLRs were performed as explained above, and intracellular measurement of cytokines was performed on day 6 in the following manner. The cultures were incubated with 25 ng/ml PMA, 1 µg/ml ionomycin, and 1 µg/ml GolgiPlug (4 h, 37°C), after which the cells were removed from the plates and washed once in PBA. The cells were permeabilized and stained with anti-IL10, anti-IFN-γ and anti-IL-4 Abs. The appropriate non-immune IgG Abs were used as isotype controls. After staining, cells were fixed in 1% PBS buffered formalin and analyzed on a flow cytometer. Region analysis was set according to forward- and side-scatter patterns; positive values were determined according to isotype controls. The data obtained was analyzed using FlowJo software (Treestar, Ashland, OR).

Detection of surface C1q with different cell preparation techniques

Whole blood was obtained from healthy donors, washed twice in PBA and non-specific binding was blocked by incubating the cells with 1 mg/ml human IgG in 100 µl PBA/1 x 10^6 cells (30 mins, room temperature). The cells were stained with anti-C1q Ab (30 mins, room temp.) After incubation the samples were washed twice in PBA, and incubated with Alexa 488 conjugated IgG F(ab')2 (30 mins, room temp), followed by treatment with

FACS/Lyse solution to lyse red blood cells (10 mins, room temp.). The cells were washed once in PBA, fixed in 1% PBS buffered formalin and analyzed by flow cytometry. Alternatively, PBMCs were isolated by Lymphoprep density gradient centrifugation and stained for flow cytometry as described above.

Detection of C1q on cell surface, in whole cell lysates and in DC culture supernatants by ELISA

Monocytes were enriched from MNC preparations by plastic adherence and cultured in 10% FBS/RPMI at a concentration of 0.5×10^6/ml supplemented with GM-CSF + IL-4. Cells were collected each day and cell lysates were prepared using a commercially available cell lysis buffer according to the manufacturer's instructions. For detection of surface-specific C1q, the cells were first surface biotinylated using sulfo-NHS-LC-biotin according to the manufacturer's instructions, followed by cell lysis. Additionally, culture supernatants were collected on days 1-4. The presence of C1q was tested in each sample by enzyme-linked immunosorbent assay (ELISA). For detection of C1q in whole cell lysates and culture supernatants, microtiter plates (MaxiSorb, Nunc, Denmark) were coated with C1q-specific mAb or the appropriate isotype-matched control at a concentration of 5 μg/ml in coating buffer (100 mM Na_2CO_3/$NaHCO_3$, pH 9.6) (2 h, 37°C). To detect surface C1q, surface biotinylated cell lysates were captured on

microtiter plates coated with streptavidin or BSA (negative control) at a concentration of 5 μg/ml. Non-specific binding sites were blocked using 3% heat inactivated (56°C, 90 min) BSA in PBS (1 h, 37°C). In our experience, we have found that even the highest grade BSA can contain trace amounts of C1q, therefore we routinely use heat-inactivated and micro-filtered BSA. Because bovine complement is unusually resistant to heat inactivation, a 90 min incubation at 56ºC is necessary to ensure destruction of C1q activity. Highly purified serum C1q was used at concentrations ranging from 1 to 5000 ng/ml as positive control and to establish a standard curve for the experiments using whole cell lysates and cell culture supernatants. Media alone was used as a negative control. Next, 100 μl of each sample were added (1 h, 37°C), followed by a C1q-specific polyclonal Ab (1 h, 37°C). For detection of the reaction, AP–conjugated rabbit anti-goat IgG was used (1 h, 37°C). All these steps were performed in ELISA buffer (PBS, 1% BSA, 0.05% Tween 20) and each step was followed by 3 washes with PBS/0.05% Tween 20. Enzyme activity was assessed by the addition of the substrate pNPP. The optical density (OD) at 415 nm was measured using a kinetic microplate reader (μQuant; Bio-Tek Instruments, Winooski, Vermont) at various time points, and the values of the negative control were deducted from the experimental values. The sensitivity of the ELISA was 5 ng/ml.

Immunofluorescent microscopy

Cell surface staining of freshly obtained mononuclear cells was performed as described above for flow cytometric analysis. Cells fixed for 5 min in 1% PBS buffered formalin were washed twice with cold PBA, concentrated by centrifugation, and applied to microscope slides using a Cytospin (Thermo Fisher). Subsequently the slides were allowed to air-dry in the dark for 5 min, and cover slips were mounted onto the slides using Immu-mount mounting solution. The slides were viewed on a Zeiss Axiovert 200 M digital deconvolution microscope, followed by image capture at 63x (oil) magnification and analysis with Axiovision 4.5 software. The results represent the analysis of 100 cells in three independent experiments. Monocytes were identified by co-staining for monocyte specific markers in addition to anti-C1q.

Statistical analysis

Student t-tests were performed using statistical software (Excel; Microsoft, Redmond, WA). A value of $p \leq 0.05$ was considered to be a significant difference. (n values represent separate experiments performed using different donors)

CHAPTER 3

RESULTS

I) Phenotypic and functional characterization of C1q treated iDCs

Exogenous C1q sustains the expression of the monocyte marker CD14 on monocyte-DCs in culture

In order to gain insight into the role of extracellular C1q during the monocyte-to-DC transition, monocytes were cultured in the presence of exogenously supplied C1q in addition to GM-CSF+IL4. In support of altered DC differentiation, monocyte-derived DCs failed to down-regulate CD14 on their surface and maintained increased expression of the molecule until day 4 in culture (Fig 2A&B). Mean fluorescence intensity (MFI) analysis revealed a 72% loss of CD14 molecules by day 1 with GM-CSF+IL-4 alone, whereas C1q treatment resulted in only a 26% loss of CD14 molecules (Fig. 2A). The pattern of increased CD14 MFI between the two culture conditions was sustained until day 4. In further support of altered differentiation, the percent of CD14+ cells was significantly higher with the addition of C1q on days 2-4 (Fig. 2B). Our analysis revealed

no differences in CD14 between the two culture conditions beyond day 4 (data not shown). Dose response analyses using C1q within physiological range (10-50 µg/ml) revealed that the effects of C1q on CD14 expression occurred in a dose-dependent fashion (Fig. 2C). To elucidate whether increased CD14 expression in the presence of C1q was in response to the effects of C1q alone or in combination with the supplied DC growth factors, we cultured monocytes in media without cytokines, with and without the addition of C1q. Intriguingly, baseline CD14 expression (day 0) increased in response to C1q, and there was a significant difference of CD14 levels between cells cultured with and without C1q on day 1 (Fig. 2D), indicating that CD14 expression is not only maintained, but increased with C1q treatment.

Altered DC differentiation in response to C1q is not due to selective DC death

In order to eliminate the possibility that the increased number of CD14+ cells developing in the presence of C1q was due to selective cell death of CD14- DCs, we monitored apoptosis by Annexin V staining on days 1-4. In Figure 3A we show that the inclusion of C1q does not increase apoptotic events on day 3. Similar results were obtained for days 1, 2 and 4. Furthermore, flow cytometric analysis of C1q treated monocyte-DCs revealed light scatter patterns consistent with viable cells, and Annexin V/propidium iodide

staining confirmed the lack of apoptosis and secondary necrosis (data not shown). Together these data confirm that C1q induced altered DC growth was not due to selective survival of CD14^{+} cells and indicate that C1q modulates the monocyte-to-DC transition at the earliest stages of DC growth (days 1-3). Through morphological analysis (Wright stain) we identified cells exhibiting typical features of iDCs, including extended membrane processes in cultures containing DC growth factors (GM-CSF+IL-4) (Fig. 3B; left panel). With the addition of C1q, cells lacked DC-like membrane processes, were smaller in size, and exhibited monocyte-like features (Fig. 3B; right panel). The monocyte-like morphology is consistent with increases in monocyte associated CD14 on these cells.

C1q inhibits the expression of monocyte-derived DC maturation markers

To further characterize the iDCs that developed in the presence of C1q, we analyzed the expression of several DC maturation-dependent markers by flow cytometry on the developing cells. C1q treatment significantly diminished CD86 expression, a required co-stimulatory molecule for T cell activation (Fig. 4A). For CD83, cells were dimly positive and MFI values were not statistically significant in the presence/absence of C1q (data not shown). Nonetheless, the percentage of CD83^{+} cells was statistically decreased by day 3

with C1q treatment (Fig. 4B). With HLA-DR, 100% of the cells were positive under both conditions, and no statistically significant differences occurred in MFI values on day 2 (Fig. 4D). Thus, even in the presence of DC growth factors, the addition of exogenous C1q at the onset of DC differentiation alters DC growth.

Figure 2. C1q sustains CD14 expression on monocyte-derived DCs in culture.

MNCs isolated from PB by density gradient centrifugation were cultured in the presence of GM-CSF+IL-4 ± 25 µg/ml C1q (A&B), or without the addition of cytokines ± 25 µg/ml C1q (D). For the dose response analysis several concentrations of C1q were added as indicated (C). Cells were analyzed on days 0-4 for the expression of CD14. (A) DCs cultured in the presence of C1q failed to down-regulate CD14 on their surface and maintained increased CD14 expression until day 4. The numbers in the upper corners of the plots represent %+ cells/ MFI. One representative experiment illustrated by dot plot analysis is shown. (B) Temporal analysis performed by flow cytometry revealed significantly higher levels of CD14 expression in C1q treated cultures (G4+C1q) compared to G4 alone. Increased CD14 levels were detectable until day 4. *p<0.05, **p<0.01 (n=4) (C) Dose response analysis confirmed that the MFI of CD14+ cells correlates positively with increasing doses of C1q on day 2. (n=3) (D) Baseline CD14 expression (day 0) on monocytes increased after 24 h of C1q treatment without the addition of DC growth factors (n=3). Cells were gated on the DR+ population for all experiments.
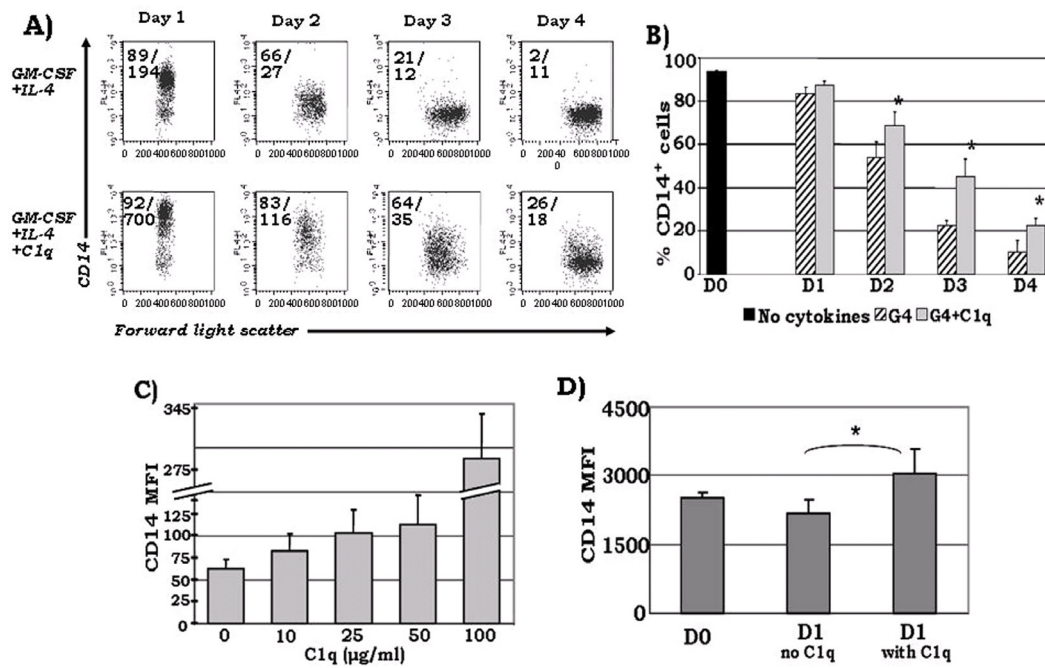
Figure 2.

Figure 3. C1q induced DC growth arrest is not due to selective DC death.

MNCs were isolated from PB and cultured with or without 25 μg/ml C1q. Cells were collected on days 1-4 and analyzed for the co-expression of CD14 and annexin V. (A) Annexin V analysis revealed that monocyte-DCs cultured in the presence of C1q did not have higher % apoptosis of CD14- or CD14+ cells than those cultured without on day 3. A typical experiment is shown illustrated by dot plots gated on HLA-DR+ cells (n=3). (B) Microscopic observation of monocyte-DCs cultured in the presence of C1q (right panel) did not show increased number of apoptotic cells by Wright stain analysis. C1q treated cells (right panel) were smaller in size and displayed monocyte-like morphology, lacking the typical extending membrane processes (arrow) characteristic of iDCs developing in the presence of GM-CSF+IL-4 alone (left panel).

A)

GM-CSF+IL-4

CD14

Annexin V

5

FL1-H

GM-CSF+IL-4+C1q

FL4-H

5.5

FL1-H

B)

GM-CSF+IL-4   GM-CSF+IL-4+C1q
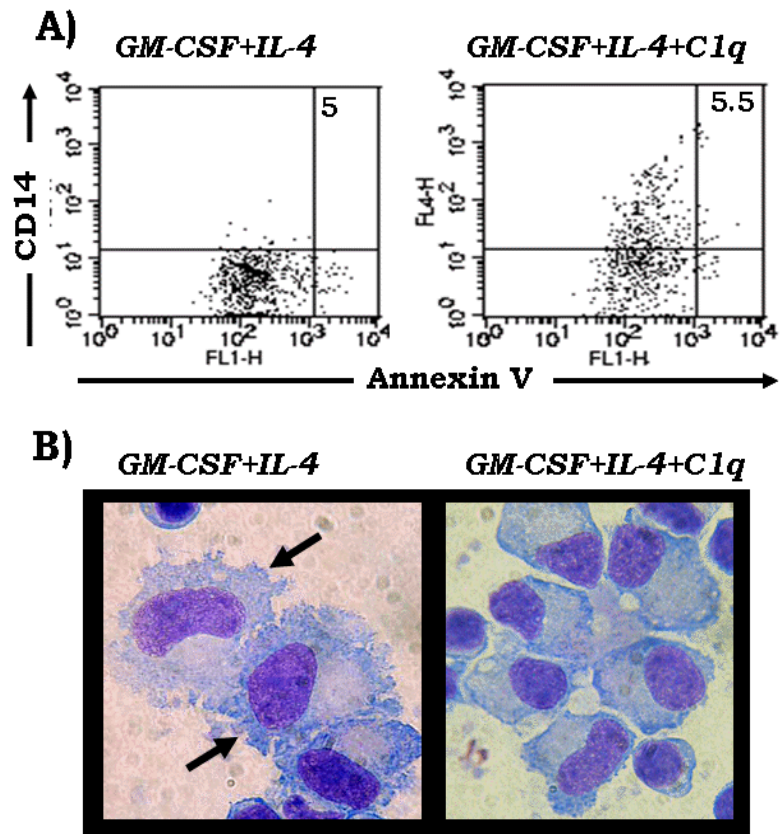
Figure 3

Figure 4. C1q delays GM-CSF+IL-4 induced DC maturation.

Monocyte-DCs were isolated and cultured in the presence of GM-CSF+IL-4 (G4) and with or without 25 µg/ml C1q (A-C). For the dose response experiments several concentrations of C1q were added as indicated (D). Cells were collected on the days indicated and analyzed for the expression of CD86, CD83 and CD11c for each condition (A-C), or alternatively, on day 2 HLA-DR analyses were performed for all C1q doses (D). (A) C1q significantly decreased CD86 expression in monocyte-DCs compared to G4. $*p<0.05$, $**p<0.01$ (n=4) (B) Monocyte-DCs cultured in the presence of C1q showed a decrease in the percentage of CD83+ cells in comparison with cells cultured in G4 alone. While CD83 expression was detected on the surface of these cells, their MFI remained low throughout the days with or without the addition of C1q (data not shown). $*p<0.05$ (n=4) (C) CD11c expression was increased by day 2 with the addition of C1q compared to day 0. There was no significant difference in CD11c expression levels on cells cultured with or without C1q. (n=6) (D) Dose response analysis revealed that the MFI of HLA-DR+ cells correlates negatively with increasing doses of C1q on day 2. While there was little or no difference in the distribution of HLA-DR on the cells at lower doses (10-25 µg/ml), at higher doses decreased expression was noted (MFI). (n=3) Cells were gated on HLA-DR+ cells for all experiments.

A) CD86

B) CD83

C) CD11c

D) HLA-DR

Figure 4.

48
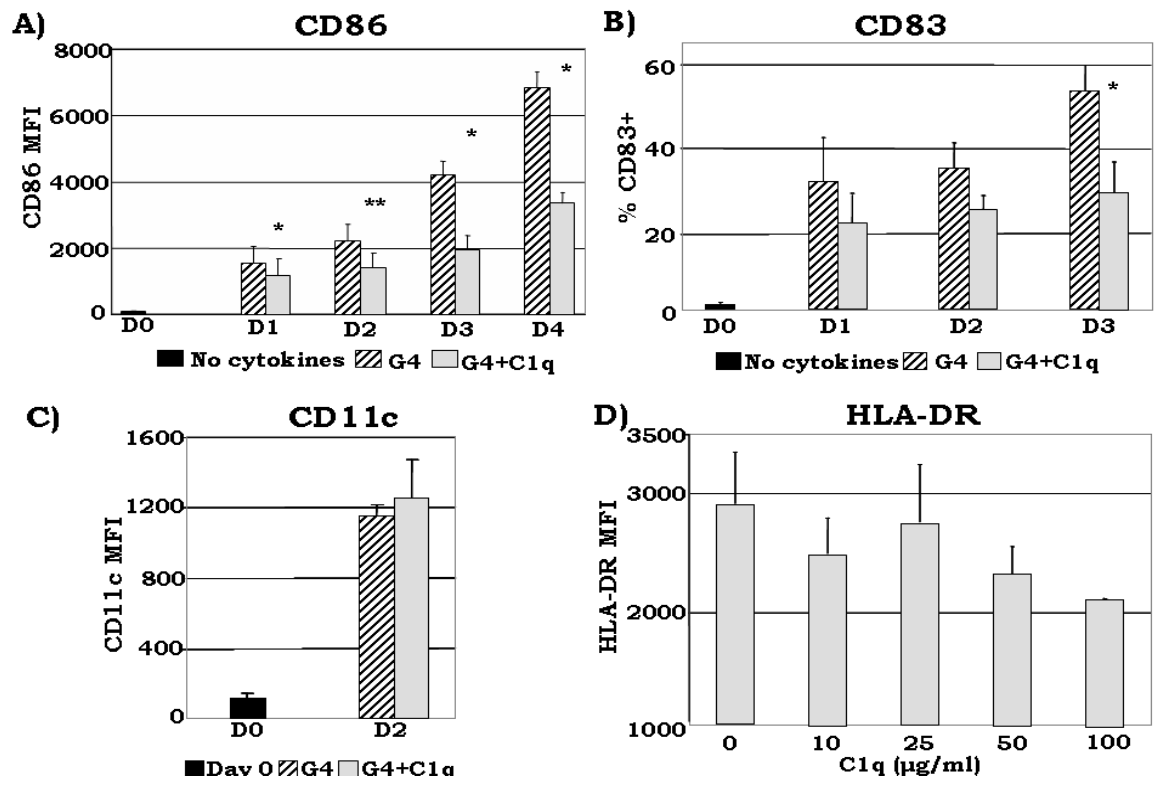
C1q promotes the development of CD14^{hi}CD11c^{hi}CD16$^{+/-}$ iDC subsets

As monocytes transition into DCs under the aegis of GM-CSF+IL-4, certain monocyte-associated molecules, including CD14 and CD16 (Fc-γ receptor III A/B) are downregulated on the cell surface. CD11c is present on PB monocytes from the outset; with GM-CSF+IL-4, progression toward the DC lineage results in the upregulation of CD11c molecules. Because we noted that C1q sustained CD14 expression (Fig 3A-C), we further studied the impact of C1q in regulating DC differentiation events by examining the developing precursor subsets. Consistent with monocyte-derived DC differentiation events, with both treatments, CD11c levels increased over time and there were no significant differences between the culture conditions (Figs. 4C & 5B). With C1q treatment CD16, like CD14, was retained on the cell surface (total CD16+ cells= 42% vs. 24% respectively on day 1). Furthermore, multi-parametric analysis revealed increased co-expression of these molecules in the presence of C1q (Fig. 5A). By day 3 CD16 levels decreased (data not shown), while CD14 levels were still elevated (Fig. 2 A&B). Because DC maturation markers were also present on C1q treated cells (Fig. 4), these data indicate that the cells acquire features of both monocyte and DC. Collectively phenotypic analysis reveals the development of CD14^{hi}CD11c^{hi}CD16$^{+/-}$HLA-DR^{hi}CD86^{dim} cells in the presence of C1q versus CD14^{-}CD11c^{hi}CD16^{-}HLA-DR^{hi}CD86^{hi} cells, which develop without C1q. The decreased expression of maturation dependent

markers on C1q treated versus untreated cells substantiates that C1q alters monocyte-to-DC differentiation.

Exogenous C1q treatment enhances the phagocytic capacity of iDCs

Since the primary function of iDCs is the uptake of self and foreign antigens, we next investigated the effects of C1q on antigenic uptake. Since opsonization of antigen and apoptotic clearance is a well described role of C1q, we sought to test uptake through complement-independent pathways in order to separate the effect of C1q as an opsonin from its effect on promoting phagocytosis of mono-DCs. The mannose receptor (MR) is a C-type lectin, which mainly binds mannosylated and fucosylated ligands present on a range of bacteria, fungi, virus-infected cells, and parasites in a C1q-independent way (Gordon, 2002). We used dextran, a bacterial polysaccharide synthesized from sucrose, to assess mannose receptor mediated phagocytosis. Lucifer yellow, a fluorescent dye taken up exclusively by pinocytosis, was used to measure receptor-independent uptake, and transferrin, essential for the import of iron, was used as a control for transferrin receptor-mediated uptake. Due to the presence of putative dextran sulfate binding sites on C1q (Hughes-Jones and Gardner, 1978), we ensured that there was no C1q in the extracellular environment during the assay by washing the cells in PBS twice to remove any remaining C1q.

Figure 5. C1q promotes the development of distinct iDC subsets.

iDCs were generated from PB cultured in the presence of GM-CSF+IL-4±25 µg/ml C1q. Cells were analyzed by flow cytometry on day 1 and 2 for the expression of CD14, CD16 and CD11c for each condition. (A) Multiparametric analysis reveals increased expansion of CD14hiCD16+/- cells on day 1&2 in the presence of C1q. These cells represent distinct subsets of iDCs. While CD14 expression was sustained until day 4; CD16 levels decreased by day 3 (data not shown). The numbers in the upper left and lower right quadrants of the plots represent %+/ MFI values of CD14 and CD16 respectively; the numbers in the upper right corners indicate %+ cells co-expressing CD14 and CD16. (n=4) (B) 100% of the cells were CD11chi with little or no difference in MFI between cells cultured with or without C1q. The numbers in the dot plots represent MFI values of CD11c. One typical experiment is shown; cells were gated on HLA-DR+ cells. (n=4)
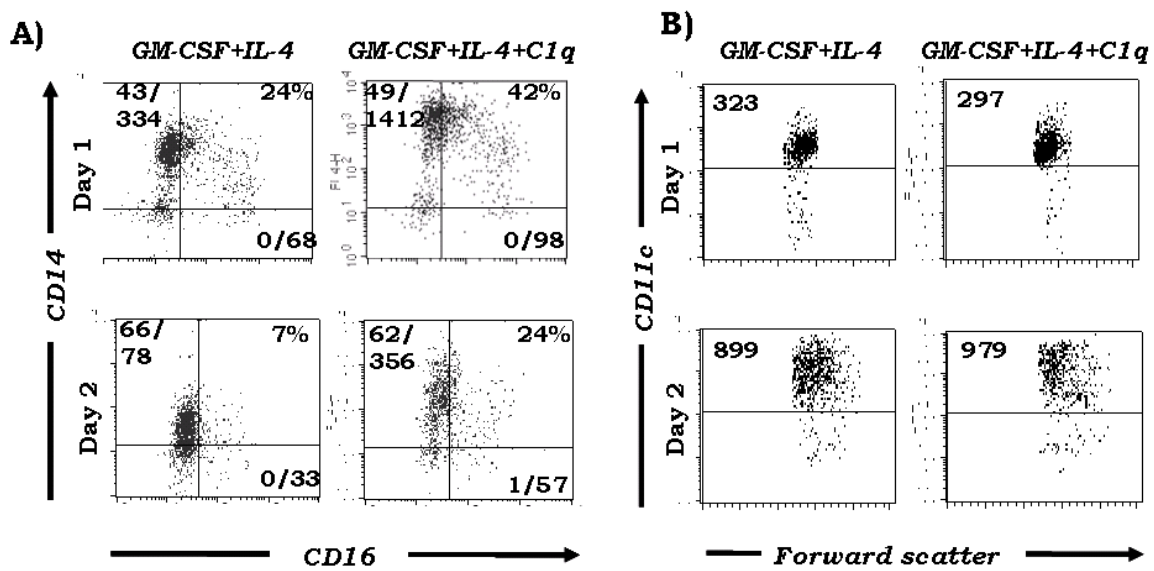
Figure 5.

Figure 6. C1q increased the phagocytic uptake in mono-DCs on day 3.

(A) C1q treatment significantly increased the phagocytic uptake of FITC-labeled dextran on day 3 compared to GM-CSF+IL-4 (G4) alone (n=7). Transferrin-receptor mediated (B) and fluid phase uptake (C) did not significantly change in response to C1q treatment. (n=3) *$p<0.05$

(A) Dextran uptake

(B) Transferrin uptake

(C) Lucifer Yellow uptake
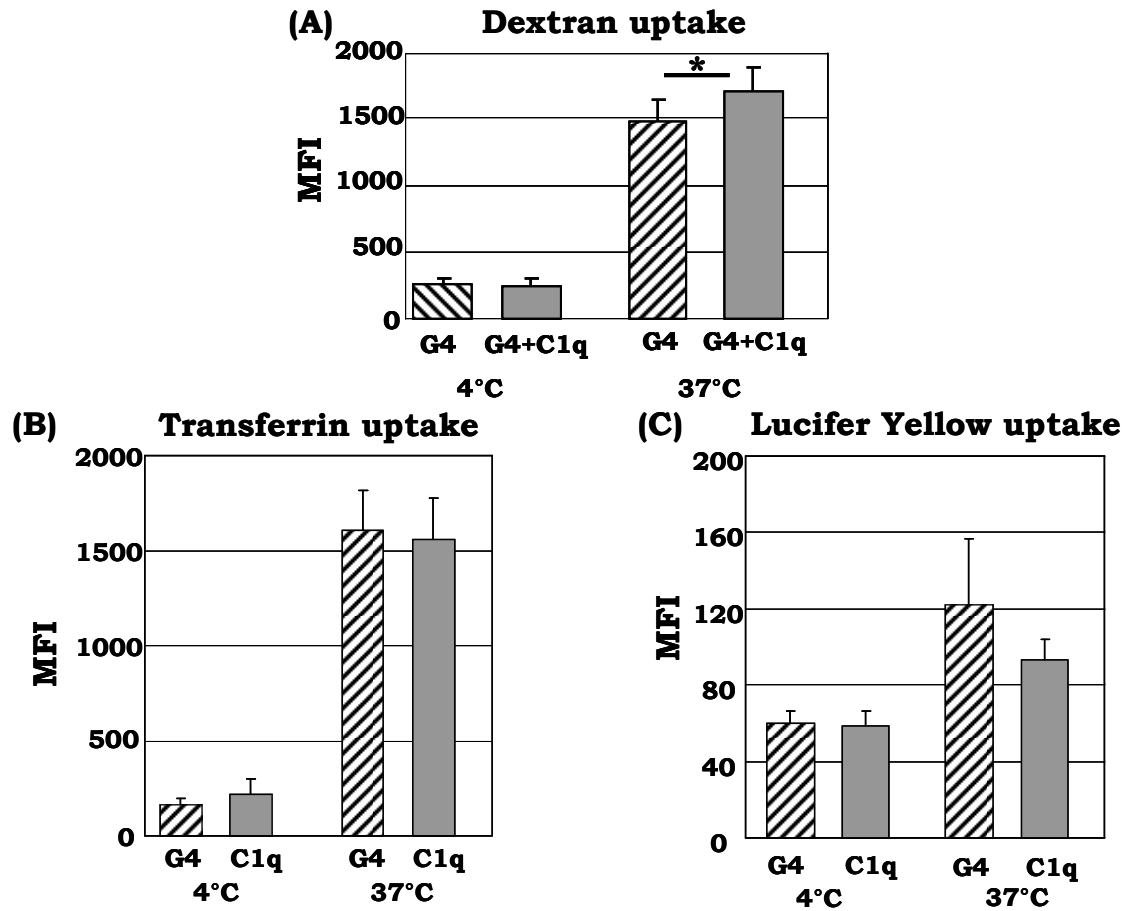
Figure 6.

As expected, cells cultured both with and without C1q exhibited low basal antigen uptake at 4°C, and all uptake levels markedly increased when the cells were incubated at 37°C (Fig. 6A-C). Furthermore, C1q treated cells demonstrated significantly enhanced dextran uptake compared to non-treated cells (Fig. 6A), while intracellular distribution of the antigen was similar with both conditions as observed by fluorescent microscopy (data not shown). Transferrin uptake (Fig. 6B) and fluid-phase mediated Lucifer yellow uptake (Fig. 6C) did not significantly change with C1q treatment, confirming that C1q does not affect these uptake mechanisms. These results suggest that C1q enhances the mannose receptor mediated phagocytic pathways without altering the internal antigen processing machinery.

C1q treatment increases the secretion, but not the production of IFN-γ, TNF-α and MIP-1α

Next we sought to correlate the secretion and the total intracellular accumulation of pro-inflammatory cytokines (IFN-γ, TNF-α, MIP-1α, MIP-1β) in the presence of C1q. To this end, relative intracellular accumulation of the cytokines was determined by flow cytometric analysis and secretion was assessed by a commercially available cytokine array after 24 hrs of cell culture. Flow cytometric analysis confirmed that cells under both conditions (+/- C1q) produced and accumulated IFN-γ and MIP-1β as was previously

reported (Fig. 7A) (Maurer and von Stebut, 2004; Yamaguchi et al., 2005). Low levels of TNF-α and MIP-1α were also detected in both cell types (Fig. 7A). However, despite similar levels of intracellular cytokine accumulation +/- C1q, secretion of both IFN-γ and MIP-1α was significantly increased in response to C1q (Fig. 7B). TNF-α also increased with C1q, however the difference was not significant during the 24h culture period (Fig. 7B). Secretions of other inflammatory-type cytokines (IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, RANTES) were similar with or without C1q (data not shown). The apparent contradiction between intracellular and secreted IFN-γ and MIP-1α by C1q treated cells implies that increased secretion does not relate to an excess of iDCs capable of producing these cytokines, but rather to an increased production or secretion per cell. Taken together, these data indicate that that C1q treated iDCs produce IFN-γ and possibly other DC differentiation/maturation specific cytokines (MIP-1 and TNF-α), which is in agreement with our immunophenotypic analysis showing that these cells are committed to the myelo-dendritic lineage. Furthermore, the selective secretion of cytokines by iDCs indicates a potentially rapid innate immune response employed by these cells in response to C1q.

Figure 7. Intracellular accumulation and secretion of cytokines during mono-DC differentiation in response to C1q treatment.

(A) Similar levels of intracellular accumulation of cytokines were detected after 24 hrs using flow cytometry +/- C1q. A protein transport inhibitor (GolgiPlug) was added to the cultures for 18h. (n=4)

(B) Secretion of IFN-γ and MIP-1α was significantly increased with C1q, while TNF-α and MIP-1β were slightly elevated. Culture supernatants (24h) were used in a commercially available cytokine array (Pierce). Data presented as mean ± SD. (n=4) *p<0.05, **p<0.01

(A)

(B)
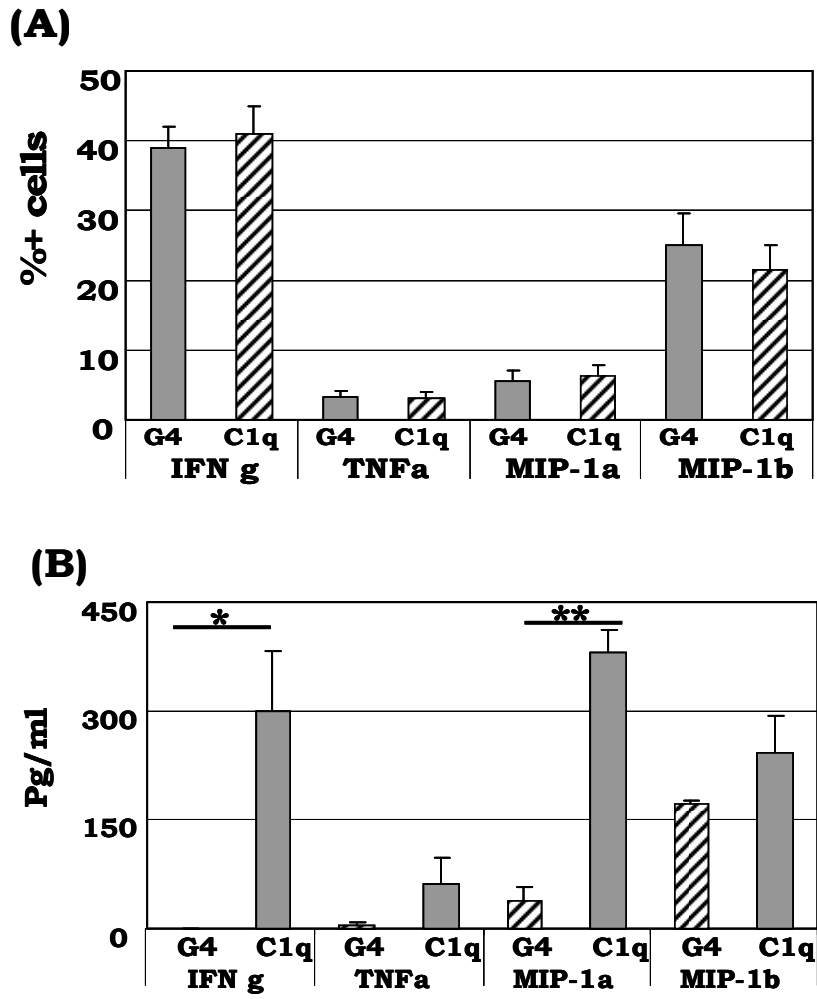
Figure 7.

C1q does not modulate iDC differentiation through IFN-γ alone

Recent work has indicated that IFN-γ can inhibit monocyte-to-DC differentiation. One such study showed that IFN-γ, a potent activator of macrophages, can modulate G4 induced monocyte-to-DC transition and shift it toward the macrophage lineage (Delneste et al., 2003). Another group demonstrated that iDCs growing in the presence of IFN-γ were maturation resistant and they promoted regulatory T cell (Foxp3+) generation (Eljaafari et al., 2009). In order to address the possibility that C1q treatment increases IFN-γ secretion by iDCs which in turn modulates iDC differentiation in our system, we cultured monocytes in the presence of IFN-γ and/or TNF-α in addition to DC growth factors. Flow cytometry assisted phenotypic analysis clearly demonstrated that exogenous IFN-γ and/or TNF-α supplied at the onset of the culture period induced the swift loss of the monocyte marker CD14 and increased the expression of CD86, HLA-DR and CD83 after 2 days in culture (data not shown). The cells were committed to the myeloid lineage as evidenced by the increased expression of the myeloid marker CD11c, and in contrast to C1q treated cells, were differentiating more rapidly than cells treated with G4 alone. These results led us to conclude that the regulatory effects of C1q during the monocyte-to-DC transition are not modulated via IFN-γ and/or TNF-α alone.

The allogeneic immunostimulatory capacity of C1q treated iDCs is analogous to that of control iDCs

In order to elucidate whether C1q treatment alters the T cell activating ability of monocyte-DCs, we performed allogeneic MLRs using a commercially available BrdU ELISA kit. Previous reports have indicated that C1q treated DCs showed an impaired ability to stimulate alloreactive T cells (Castellano et al., 2007b). However, these studies were performed on mature DCs after LPS stimulation. While antigen presentation and T cell stimulation are characteristic of mature DCs, iDCs also have the capacity—though to a lesser extent — to induce T cell responses either by secreted factors or by direct cell contact. Our results indicate that on day 3 C1q treated iDCs induce similar level of T cell proliferation as those treated with G4 alone (Fig. 8). Cells growing with or without C1q were able to stimulate T cells to a higher extent than the M-CSF treated macrophages. This finding was intriguing, since our results so far have indicated that C1q treatment modulates DC differentiation and induces the development of iDCs with monocyte-macrophage-like (non-APC-like) characteristics. In particular, the reduction of the co-stimulatory marker CD86 in response to C1q suggested that C1q treated iDCs might have decreased influence on T cell priming. In contrast, C1q treated iDCs showed similar or slightly increased T cell stimulation compared to control iDCs (Fig. 8).

Some studies indicate that immature and mature DCs, as well as different iDC subsets induce differential T cell responses (Jonuleit et al., 2000). Moreover, T cells stimulated by iDCs may lose their ability to produce IFN-γ, IL-2, or IL-4 (Jonuleit et al., 2000). To test this in our system, we examined T_H1/T_H2 subset distribution during MLR using intracellular flow cytometric analysis of IFNγ (T_H1) and IL-10/IL-4 (T_H2). Our results indicate that C1q treated iDCs favor the generation of similar T cell subsets as those treated with DC growth factors alone (Fig. 9A). Similarly to previously described results (Santiago-Schwarz et al., 2001), iDCs in our system generated a mixed $T_H1/T_H2/T_H0$ response, with the majority of the cells secreting IL-4 and IFN-γ in both cultures (T_H0) (Fig. 9B). The data indicate that while C1q regulates the differentiation and certain functions of iDCs, allogeneic T cell priming abilities remain similar to untreated cells. Taken together, our results suggest that in the absence of danger signals C1q may help maintain steady state conditions by skewing DC differentiation toward a 'hybrid' cell type with both monocyte-macrophage-like (increased CD14, enhanced phagocytosis, IFN-γ secretion) and DC-like (T cell priming) characteristics.

Figure 8. C1q treated iDCs were capable of allostimulation to a similar or greater degree than iDCs treated with GM-CSF+IL-4 alone.

Stimulator cells were cultured for 3 days in G4 +/- C1q, removed from culture and adjusted to equal concentrations in 10% FBS/RPMI. Responder cells were obtained by isolating Nylon wool-enriched T cells from normal PB, than mixed with stimulators in 1:2, 1:4, 1:8 (stimulator : responder) ratio/well in U-bottom 96-well plates. M-CSF treated cells were used as non-APC control; stimulator or responder cells alone were used as non-proliferating control. Proliferation was measured on days 6–7 by colorimetric BrDU assay (Roche; Indianapolis, IN) according to the manufacturer's instructions. Stimulation index (SI) was calculated as (BrDU labeling index of mixed cells)/(BrDU labeling index of responder cells alone). One representative experiment is shown. (n=3)
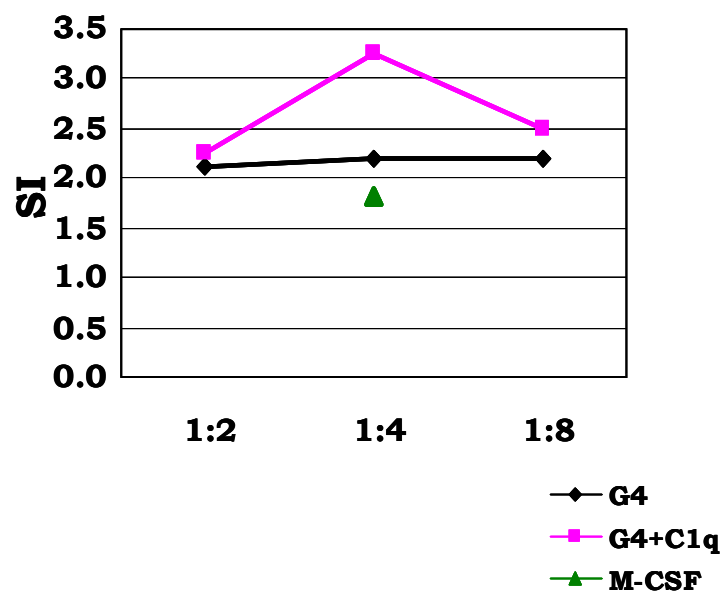
Figure 8.

Figure 9. C1q treated iDCs induce similar T cell responses as control iDCs.

(A) C1q treated iDCs induced the development of similar T cell subsets as those treated with DC growth factors alone. Mixed leukocyte reactions were performed, and T helper subset distribution was analyzed using intracellular accumulation of IFN-γ, IL-4 and IL-10 by flow cytometry. Isotyope controls were subtracted form the values. (n=2) (B) Immature DCs growing with or without C1q favored the development of a mixed $T_H1/T_H2/T_H0$ response. The dot plot analyses shown are representative of both experiments performed. (n=2)
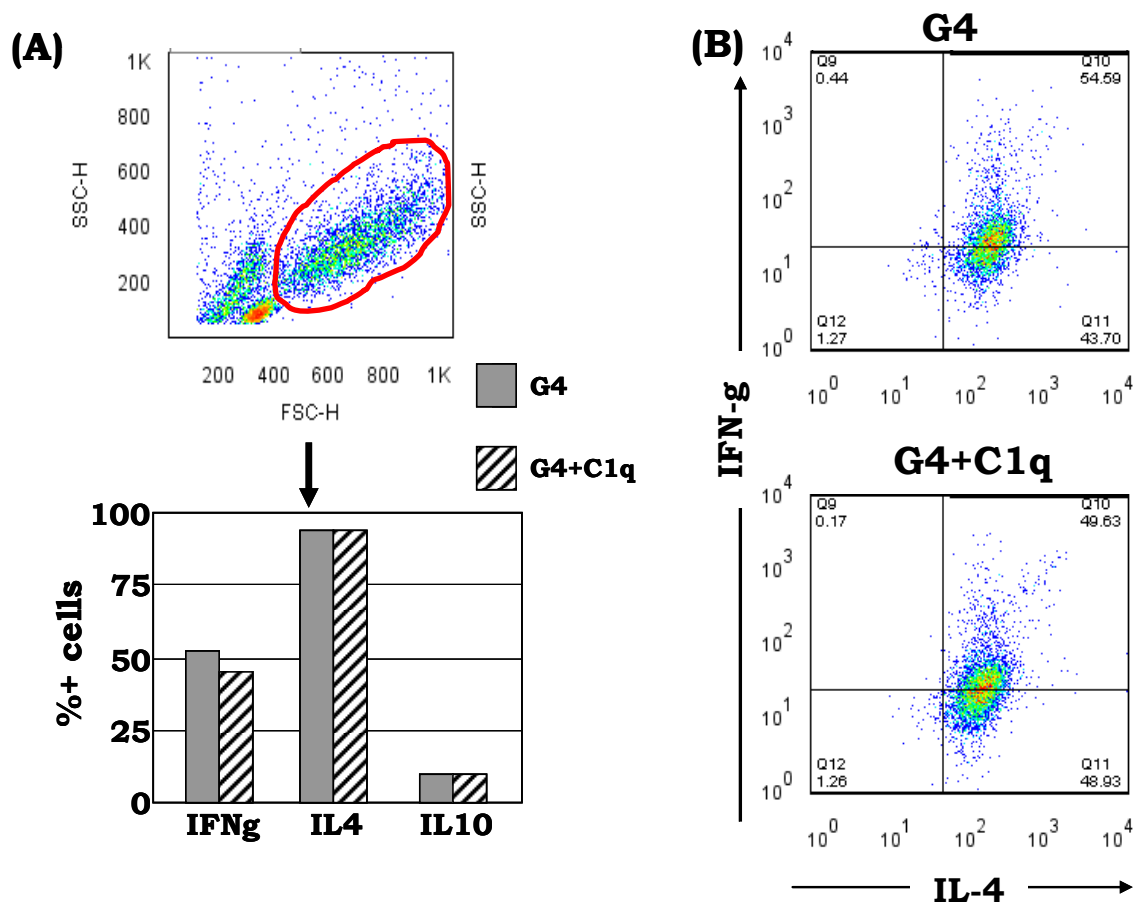
Figure 9.

II) The influence of specific C1q/C1qR interactions on early DC differentiation and function

C1q is present on the surface of human monocytes and iDCs

To further investigate the role of C1q in regulating DC activity, we analyzed its surface expression and secretion during the monocyte-to-DC transition. Surprisingly, antigen-capture ELISA experiments using whole cell lysate prepared from surface biotinylated cells revealed the expression of C1q on the surface of human monocytes (Fig. 10A). Isotype-matched non-immune Ab was used as control. Flow cytometric analysis confirmed the presence of C1q on the surface of monocytes using different cell preparation techniques (Fig. 10B). For these experiments antibody staining was performed directly in fresh whole blood followed by red blood cell lysis, and after mononuclear cell isolation using density gradient centrifugation (MNC). In subsequent immunofluorescence microscopy experiments we used two different polyclonal antibodies to the whole C1q molecule to observe surface C1q expression after isolation of MNCs from whole blood. Analysis of surface C1q on monocytes using these Abs revealed a punctate pattern evenly distributed over the cellular membrane (Fig. 10C; left panel). Similar results were obtained for both antibodies, whereas the isotype control showed no staining (Fig. 10C; right panel). Furthermore, antigen-capture ELISA using

whole cell lysates of monocytes revealed higher amounts of total C1q than on the cell membranes alone, indicating the presence of intracellular pools of C1q (Fig. 10D).

Temporal analysis of surface C1q revealed that surface expression remains elevated until day 1, followed by a rapid decline on days 2-3, coinciding with firm commitment to the DC lineage on day 3 (Fig. 10E). C1q surface expression could be sustained beyond day 3 when monocytes were cultured with M-CSF, a mono-macrophage specific growth factor (Fig. 11G). This is consistent with previously published results showing that C1q is present on the surface of macrophages (Kaul and Loos, 2001). Despite decreasing cell surface expression between days 1-4, C1q levels remained relatively high in cell free supernatants during this period (90 ± 3 ng/ml) (Fig. 10F).

Regulation of DC maturation by C1q may depend on the varied expression of C1q receptors

Based on our results so far, we postulated that endogenously produced C1q, in concert with C1qRs, regulates DC differentiation. Here, we investigated which C1qR might be associated with the observed effect of C1q on DC growth during the monocyte-to-DC transition. To this effect we analyzed the expression of two C1qRs: gC1qR and cC1qR on monocyte-DCs cultured in the presence of GM-CSF+IL-4. On day 0 nearly all monocyte-

derived DCs expressed gC1qR (Fig 11B), while cC1qR was more variable within the population (Fig. 11A), despite the consistently elevated expression of its putative surface partner, CD91 (Fig. 11C). Even though there was a modest reduction in $gC1qR^+$ cells by day 4 (Fig. 11B), the percent of cC1qR expression increased compared to day 0 (Fig. 11A). Furthermore, MFI analysis revealed that the amount of cC1qR was dramatically amplified after day 2 (Fig. 11D), whereas the amount gC1qR remained at relatively steady levels (Fig. 11E). Thus, at the precise period (~day 3) corresponding to firm commitment to the DC lineage there is an inverse correlation between gC1qR and cC1qR expression on the cell surface, which may influence the nature and specificity of the cells' response to C1q. Interestingly, despite the increase in cC1qR expression, its surface partner, CD91, was gradually reduced during the culture period, indicating that alternate partners for cC1qR are present upon commitment to the DC lineage (Figs. 11 C & F).

Next we sought to uncover whether the distinct pattern of C1qR expression was associated with the binding orientation of C1q on the surface of monocytes and iDCs. To this end we utilized monoclonal antibodies specific to the globular head regions of C1q as well as polyclonal antibodies to the whole protein, and monitored C1q binding by flow cytometry. In agreement with our previous results (Fig. 10), pAb binding confirmed that fresh monocytes express C1q (>75%) (Fig. 11G). Furthermore, compared to baseline C1q levels (day 0), M-CSF treated monocyte-macrophages sustained surface C1q expression, while in the presence of DC growth factors C1q levels showed a 50% decrease by day 3 (Fig. 11G). In contrast, surface C1q was detected on monocytes and iDCs at much lower

Figure 10. C1q is present on the monocyte surface.

(A) Antigen-capture ELISA experiments revealed the expression of C1q on the surface of human monocytes. Whole cell lysate (WCL) prepared from surface biotinylated cells were used. (n=2) (B) Flow cytometric analysis confirmed the presence of C1q on the surface of monocytes using different cell preparation techniques. (n≥3) (C) Detection of surface C1q on fresh PB monocytes by immunofluorescence microscopy. Isotype-matched Ab was used as a negative control (n=3). (D) Monocyte derived WCLs contained higher amounts of total C1q than on cell membranes alone using antigen-capture ELISA. (n=1) (E) Cells were analyzed by flow cytometry for surface bound C1q on days 0-4. Cell surface expression of C1q was highest on days 0-2, and it was greatly reduced by day 4. Isotype-matched Ab was used as a negative control; cells were gated on the HLA-DR+ population. (n=4) (F) Cell supernatants were assessed for secreted C1q by sandwich ELISA. C1q secretion remained at a steady level of 90 ng/ml ± 3 throughout days 1-4. (n=4).
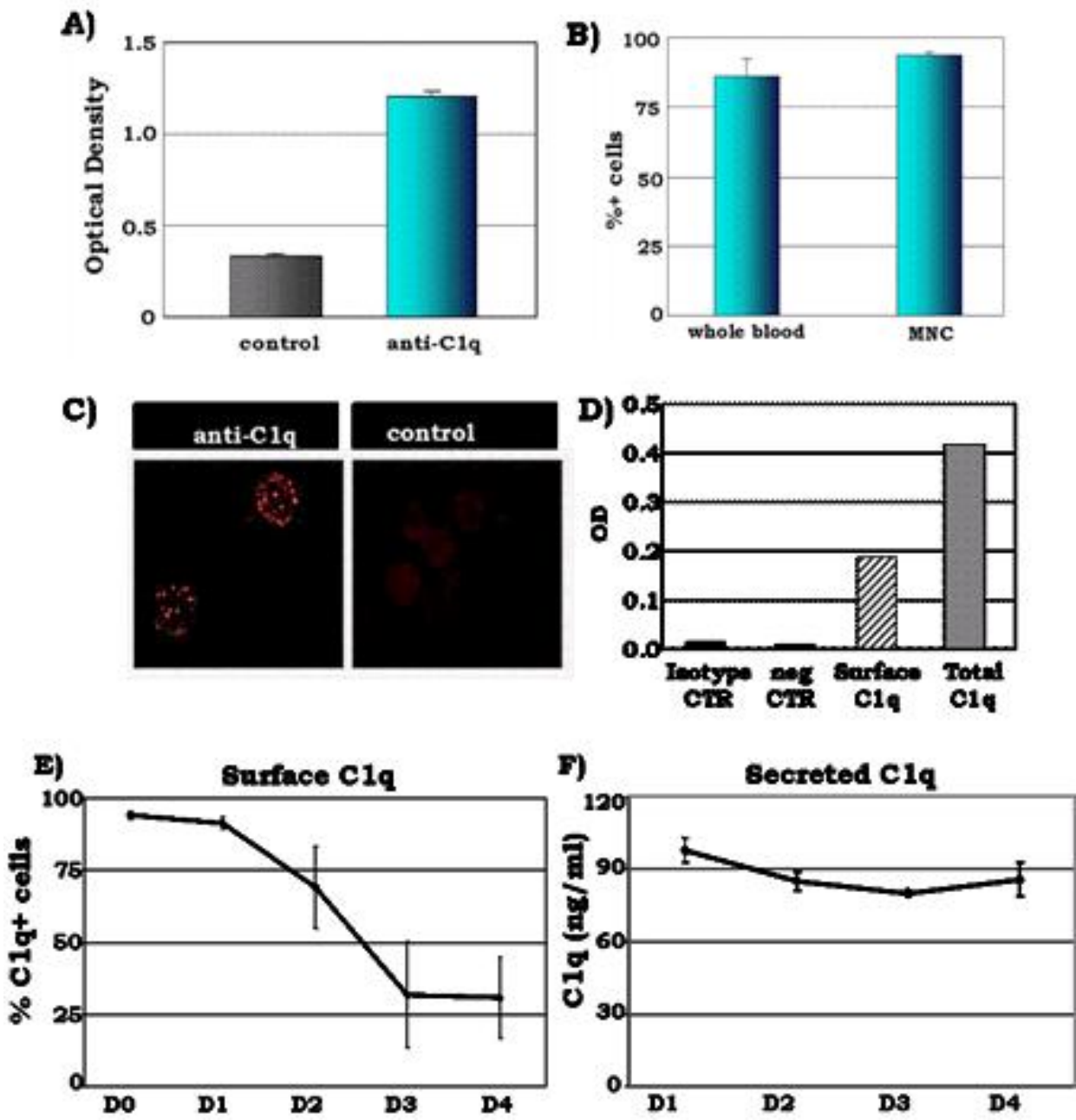
Figure 10.

Figure 11. C1q receptors show varied expression on mono-DC precursors.

MNCs were cultured in the presence of GM-CSF+IL-4, and collected and analyzed on days 0-4 for the expression of gC1qR (B&E), cC1qR (A&D) and CD91 (C&F) for both percent expression (A-C) and MFI (D-F). (A) The percent of cC1qR expression was variable on monocytes, but by day 2 nearly all monocyte-DCs had the receptor on their surface. (B) On day 0, gC1qR was present on almost all the cells, and its expression was only slightly reduced by day 4. (C) Monocytes expressed CD91 on their surface, but the percentage of CD91+ cells was significantly reduced by day 3 and 4. (D) Mean fluorescence analysis revealed that cC1qR expression was dramatically amplified by day 3 and 4. (E&F) gC1qR and CD91 MFIs remained at relatively steady levels throughout the days. (G) C1q is bound to the monocyte and DC surface via its globular head regions, while on M-CSF treated monocyte-macrophages its orientation is reversed. Binding orientation of C1q was determined using monoclonal antibodies specific to the globular head regions of C1q as well as polyclonal antibodies to the whole protein, and assessed by flow cytometry. (n=3) Experiments were gated on DR+ cells. *$p<0.05$, **$p<0.01$ (n≥4)

Figure 11.

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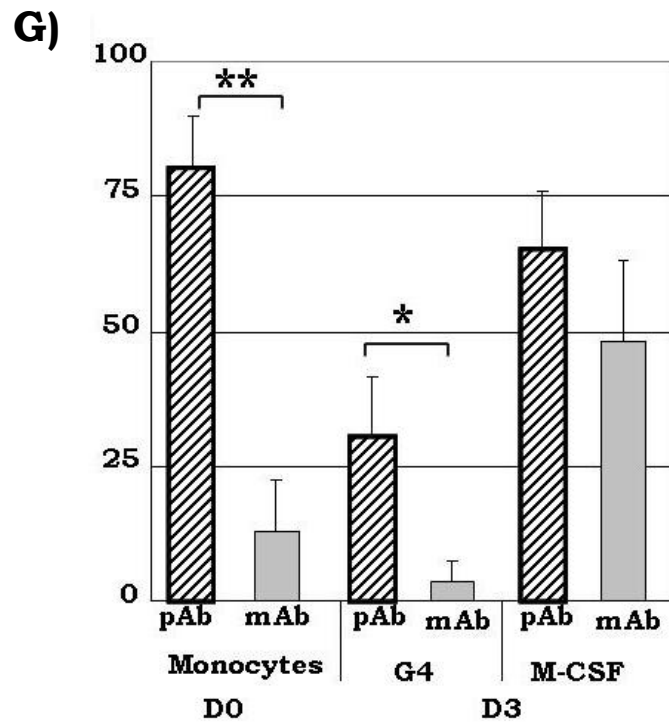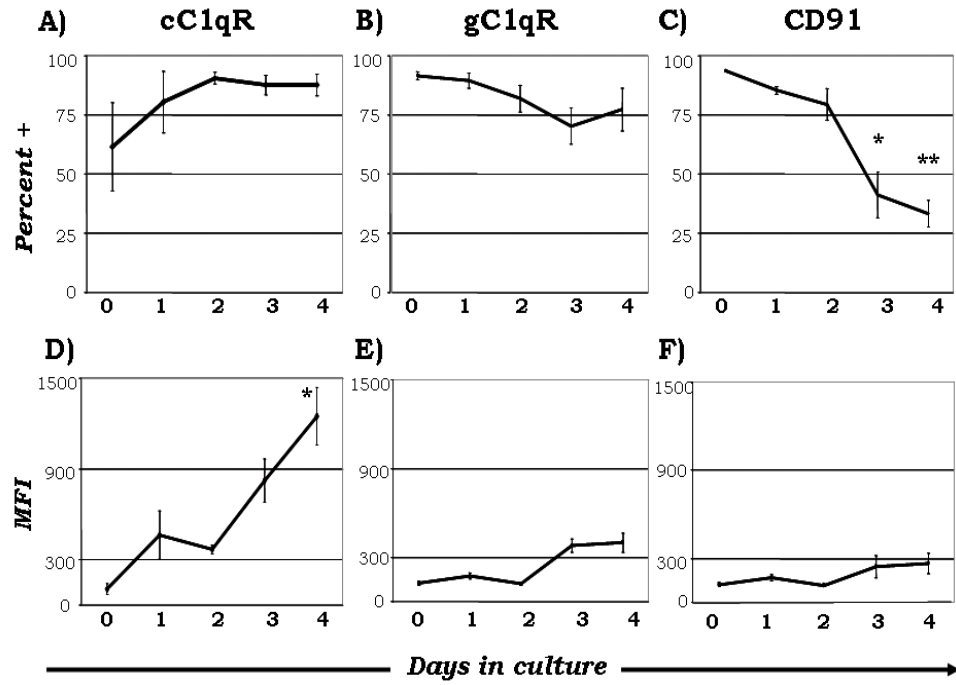
levels (2.5-10%) using mAbs specific to the globular heads, suggesting that C1q might be bound to these cells via its globular head regions (Fig. 11G). In contrast, the M-CSF treated cells showed strong binding to the mAb, confirming that C1q is displayed on these cells with the globular regions exposed, as previously described (Kaul and Loos, 2001). Taken together, these data suggest that the regulatory effects of C1q on DC differentiation and function depend on specific C1q/C1qR interactions. Additionally, consistent with the innate nature of C1q, cytokines that support innate immune functions (e.g. M-CSF) may also sustain C1q expression on monocytes.

C1 complex may be present on the surface of human monocytes

Since most of the C1q – save approximately 20% - circulates in plasma as part of the pentameric C1 complex ($C1q_1C1r_2-C1s_2$), we also considered the possibility that the C1q detected on the monocyte surface may be part of the C1 complex. To test this premise, monocytes were analyzed for surface expression of C1s and C1r, using flow cytometry. In addition, two other complement related proteins, Factor B and C1 inhibitor, were assessed as well. As Figure 12 shows, in addition to C1q, nearly all monocytes were positive for the presence of both other subunits of the C1 complex. Therefore, potentially all circulating C1q in plasma (70-160 μg/ml) can bind to monocytes, regardless of whether it is in its free form, or associated with the C1 complex. We hypothesize that circulating C1q is captured by C1qR present on the monocyte surface. In the C1 complex

only the globular heads of C1q are available for binding, while the collagen tail is masked by the associated C1 subunits. Therefore the presence of C1 complex on monocytes supports our hypothesis that C1q/gC1qR interaction is a requisite of early C1q signaling on these cells. The presence of C1 inhibitor on monocytes (Fig. 12) ensures that unwanted classical complement activation could not take place through the surface bound C1 complex. Factor B, a complement protein synthesized by monocytes (Hogasen et al., 1995), was also found on the cells.

Antibodies against gC1qR mimic the effects of C1q on mono-DC differentiation

Various proteins and antibodies that bind to gC1qR at the C1q binding site have been shown to stimulate signaling and C1q-like effects on T cells and several cell lines (Yao et al., 2003; Yao et al., 2001a). We employed this strategy to evaluate DC differentiation events mediated by gC1qR, and assessed DC growth in the presence of F(ab)'$_2$ fragments of the monoclonal Ab 60.11, which is directed against the C1q binding site on extracellular gC1qR. Similar to the effects of C1q, immunophenotypic analysis revealed that monocyte-derived DCs treated with 60.11 retained the monocyte marker CD14 on their surface. Mean fluorescence intensity of CD14 was elevated by day 1 with 60.11 (Fig. 13A), and in support of altered DC differentiation, the cells retained CD14 on their

surface until day 3 (Fig. 13A&B). Treatment with 60.11 also reduced CD86 expression (Fig. 13C), and the percentage of the activation-associated co-stimulatory marker CD83 was significantly decreased by day 2 (Fig. 13E).

Consistent with monocyte-derived DC differentiation events, by day 2 both HLA-DR and CD11c expression increased with and without 60.11 compared to baseline (day 0). Furthermore, there was no significant difference in CD11c or HLA-DR expression levels on cells cultured in the presence or absence of 60.11 (data not shown), confirming that the cells under both conditions are committed to the DC lineage. With C1q treatment CD16, like CD14, was retained on the cell surface (Fig. 5). Similarly, culturing the cells in the presence of 60.11 resulted in the elevation of CD16 by day 1 (Fig. 13D), and the increase in the percentage of cells co-expressing CD14 and CD16 (Fig 13F). By day 3 CD16 levels diminished, while CD14 levels were still significantly elevated (Fig. 13 B&D). Thus, the addition of 60.11 and subsequent ligation of gC1qR at the onset of mono-DC transition alters DC differentiation/maturation, even in the presence of DC growth factors. These data indicate that similar to the effects of C1q, gC1qR ligation by mAb specific to the C1q binding site induces the development of $CD14^{hi}CD11c^{hi}CD16^{+/-}HLA-DR^{hi}CD86^{dim}$ cells. Taken together, the data support that gC1qR plays a putative role in the C1q mediated effect on DC growth during the monocyte-to-DC transition and regulates DC differentiation by favoring the development of specific precursors.

Figure 12. All subunits of the C1 complex are present on the surface of monocytes.

Fresh peripheral blood monocytes were surface stained for C1q, C1s, C1r, C1 inhibitor (C1INH) and Factor B and analyzed by flow cytometry. (n=3)
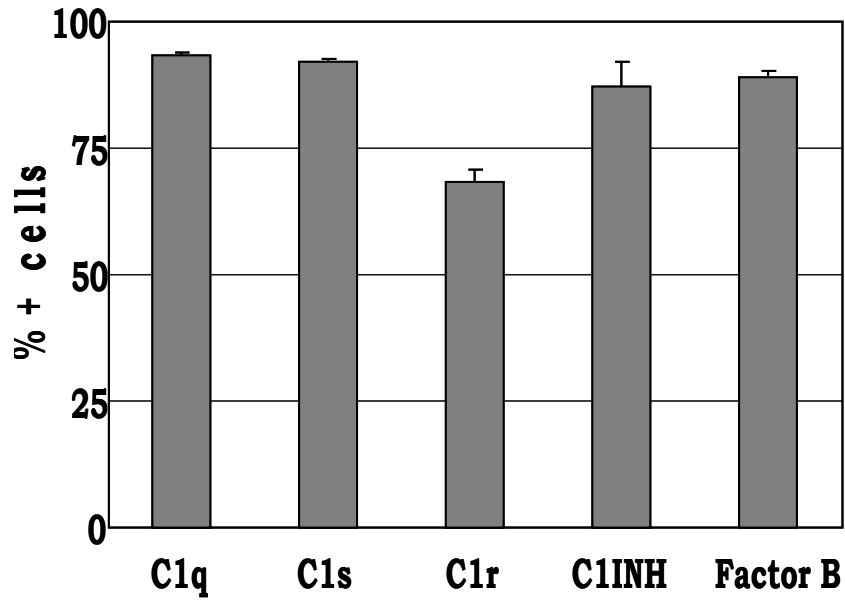
Figure 12.

Figure 13. Activating antibodies against gC1qR (60.11) alter DC differentiation events and promote the development of distinct precursor subsets.

(A) Mean fluorescence intensity (MFI) of CD14 expression increased on 60.11 treated monocyte-derived DCs in culture by day 1. Increased CD14 levels were detectable until day 3. The numbers in the upper left corners represent MFI. One representative experiment illustrated by dot plot analysis is shown. (B) Temporal analysis performed by flow cytometry showed that CD14 expression remained significantly elevated until day 3 in the presence of 60.11 (G4+60.11) compared to GM-CSF+IL-4 (G4) alone. (C) 60.11 decreased CD86 expression in monocyte-DCs compared to G4. (D) Flow cytometry assisted analysis revealed an increase in CD16+ cells in the presence of 60.11 on day 1. CD16 expression decreased by day 3. (E) Monocyte-DCs cultured in the presence of 60.11 showed a decrease in the percentage of CD83+ cells in comparison with cells cultured in G4 alone. CD83 was dim on both cultures indicating that its expression was not due to the presence of endotoxin. (F) Multiparametric analysis showed increased expansion of CD14hiCD16+/- cells by day 1 in the presence of 60.11. These cells represent distinct subsets of iDCs. The numbers in the upper left and lower right corners of the plots represent total %+ cells/ MFI. The numbers in the upper right corners represent %CD14+CD16+ cells. For all experiments cells were gated on HLA-DR+ cells. (n=3) *p<0.05
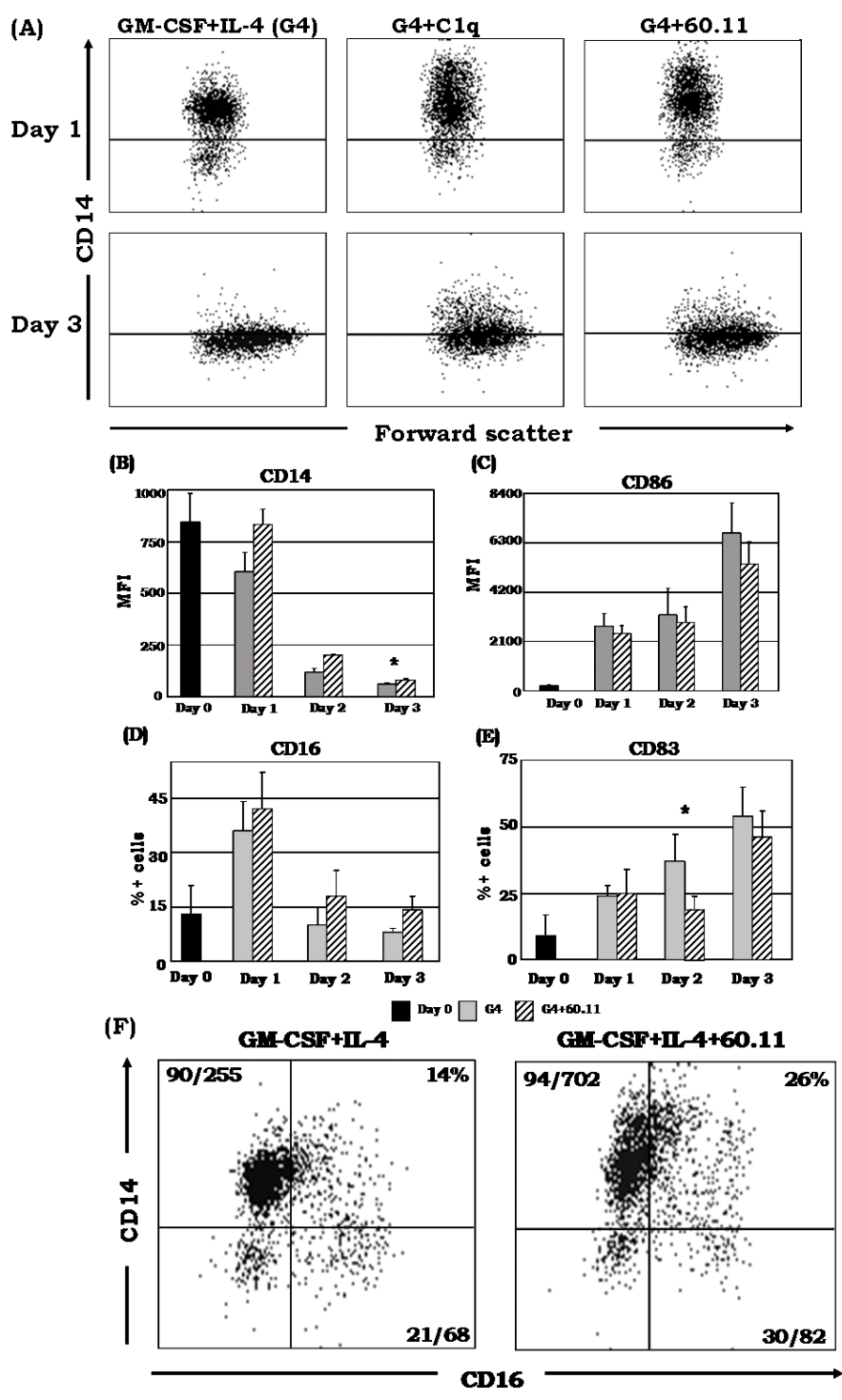
Figure 13.

CHAPTER 4

DISCUSSION

C1q is a multifunctional, highly structured molecule that recognizes injurious stimuli such as foreign antigens, cell debris and apoptotic cells, and regulates the innate immune system by mediating their removal by phagocytosis (Maniati et al., 2008; Paidassi et al., 2008). Similar to the role of other innate immune system molecules, C1q can also induce inflammatory responses that are associated with adaptive immunity (Fraser et al., 2006; Kishore et al., 2004; Wallach et al., 1999).

The work presented here was performed to test the hypothesis that locally synthesized C1q plays a fundamental role in regulating DC differentiation and function during the monocyte-to-DC transition with special emphasis on the role of C1q/C1qR interactions. Our results show that C1q modulates GM-CSF+IL4 induced DC differentiation, as evidenced by retention of monocyte markers and reduced expression of DC maturation and co-stimulatory molecules. C1q treatment resulted in significantly increased secretion of IFN-γ and MIP-1α after 24 hours, and significantly enhanced the phagocytic uptake capacity of the cells on day 3, while it did not change their allogeneic immunostimulatory capacity. Taken together, these data suggest that in the absence of

danger signals C1q may help maintain steady state conditions by skewing DC differentiation toward cells with monocyte-macrophage-like characteristics.

For the first time we show here that freshly isolated peripheral blood monocytes carry C1q on their surface. The binding pattern of a monoclonal antibody specific to the globular heads of C1q indicated that C1q is bound to monocytes and iDCs via its globular heads, presumably through gC1qR, the receptor for the globular heads of C1q. Culturing monocyte-DCs in the presence of a monoclonal antibody recognizing the C1q binding site on gC1qR resulted in the development of cells similar to those with C1q treatment. These results indicate that C1q/gC1qR interaction is a requisite of early C1q signaling on these cells.

Our results suggest that soluble C1q that is free of antigenic or apoptotic 'cargo' may serve as an autocrine signal that regulates differentiation of monocytes into DCs, as evidenced by retention of CD14 and reduced expression of DC maturation markers and co-stimulatory molecules. Possibly the increased CD14 levels on C1q treated mono-DCs results from upregulated transcription or translation events. Alternatively, since CD14 is a GPI anchored molecule that is routinely shed into the extracellular milieu, the kinetics of shedding may be delayed or reduced. C1q did not operate by inducing selective apoptosis of CD14^{neg} DCs, as is the case with IL-10 (Chang et al., 2007), suggesting that C1q alters DC differentiation via a separate pathway than IL-10. The effect of C1q was rapid (within 1-2 days) and was most efficient during the earliest stages of DC growth.

In support of a role for C1q in regulating DC differentiation, is that addition of exogenous C1q together with DC growth factors resulted in the development of an "interface cell" (CD14^{hi}CD11c^{hi}CD16$^{+/-}$HLA-DR^{hi}CD86^{dim} cells) exhibiting features of both monocytes and iDCs. Interestingly, similar subsets have been linked to certain inflammatory disease processes. The subset of CD14^{hi}CD16^{+} iDCs has been described to exhibit characteristics of both monocytes and DCs, possibly enabling these cells to activate innate, as well as adaptive immune responses (Grage-Griebenow et al., 2001a; Grage-Griebenow et al., 2001b; Passlick et al., 1989). Aside from elevated IL-12 and TNF-α production and high expression of T cell stimulatory molecules, these cells display high phagocytic activity and retain CD14 on their surface, indicating signaling potential via monocyte- and macrophage-associated receptors (Grage-Griebenow et al., 2001a; Grage-Griebenow et al., 2001b; Ziegler-Heitbrock, 2007). Interestingly, while there are physiologic conditions in which this subset is expanded to >20% (Scherberich and Nockher, 1999; Ziegler-Heitbrock, 2007), the precise role of these cells in immunopathogenic events is unclear.

C1q treatment resulted in a small, but significant elevation in the phagocytic capacity of iDCs as assessed on day 3 by dextran uptake. Because the unaltered CD11c and MHC class II expression on C1q treated iDCs indicate that the cells are committed to the DC lineage, we hypothesize that increased uptake is due to a direct enhancement of phagocytic function, rather than to a delay in the DC differentiation process. Interestingly, a novel antigen transport function has been recently described for the MR,

targeting antigens to peripheral lymphoid organs for clearance (Allavena et al., 2004). This function allows the MR uptake of potential auto-antigens from plasma and extracellular compartments, thereby contributing to the autoimmune disease process. Based on our results we predict that a C1q deficient environment results in DCs deficient in phagocytosis. In fact, monocyte-derived macrophages obtained from C1q deficient patients showed a kinetic defect in the phagocytic uptake of apoptotic cells in vitro (Taylor et al., 2000). Antigen uptake in the absence of danger signals has been shown to result in DC tolerance (Shimizu and Fujii, 2008), thereby potentially silencing the immune system to self-derived antigens and preventing unwanted immunologic activation. In contrast, the predicted decrease in phagocytic capacity of C1q deficient DCs may induce a self-directed adaptive immune response and contribute to the autoimmune disease process.

C1q treated cells in our system secreted increased amounts of IFN-γ into the extracellular milieu. The production of IFN-γ by APCs has been a controversial issue. While the notion of myeloid production of IFN-γ has been met with skepticism, recent work has addressed these concerns and showed that APC-derived IFN-γ plays an important role in the initial stages of infection (Frucht et al., 2001). The effect of cytokines is highly dependent on the context they are produced in; their concentration, the microenvironment, the timing of production, etc. will highly influence T helper cell differentiation toward the T_H1 (innate) or T_H2 (adaptive) cell type. IFN-γ directly promotes the development of a T_H1 phenotype by positive feedback. On the other hand,

through an indirect, negative regulatory mechanism, IFN-γ can control the regulation of the homoeostatic balance between T_H1 and T_H2 cells, thereby supporting a T_H2 response (Rissoan et al., 1999). While a predominantly pro-inflammatory cytokine, endogenous IFN-γ may also have the potential to play a role in preventing autoimmune disease (Wood et al., 2007). In certain autoimmune disorders, reduced IFN-γ secretion has been implicated in the pathogenesis of disease. Interestingly, consequent studies have shown that although mononuclear cells from diseased individuals were able to produce intracellular IFN-γ, they had a reduced ability to secrete this cytokine (Tang and Kemp, 1994). These observations may be of particular relevance, since in our experiments C1q treatment increased the secretion, but not the production of IFN-γ by mono-DCs in vitro.

The data indicating that C1q increases the production of IFN-γ and possibly other DC differentiation/maturation specific cytokines (MIP-1α and TNF-α) are in agreement with our immunophenotypic data showing that C1q treated cells are committed to the myelo-dendritic lineage. Increased IFN-γ production suggests that the developing cells may favor T_H1-type responses. IFN-γ is known to increase TNF-α secretion, which in turn inhibits production of IL-10 (Paludan, 2000). MIP-1α, a chemokine involved in inflammatory host responses, as well as regulation of tissue homeostasis, can also upregulate the release of TNF-α (Maurer and von Stebut, 2004). Since TNF-α levels increased steadily as culture time progressed (data not shown), IFN-γ and MIP-1α driven secondary cytokine production may account for the high levels of TNF-α in the C1q treated cultures. IFN-γ and TNF-α are well known to work synergistically and produce an

enhanced innate immune response (Paludan, 2000). Based on these observations we postulate that C1q may selectively trigger the secretion of IFN-γ and other cytokines from intracellular pools. Selective secretion of cytokines in response to C1q by iDCs would allow for rapid enhancement of the innate immune response, and may help establish a link between innate immunity and the developing adaptive immune response.

Recent work has indicated that IFN-γ can inhibit monocyte-to-DC differentiation. One such study showed that IFN-γ, a potent activator of macrophages, can modulate G4 induced monocyte-to-DC transition and shift it toward the macrophage lineage (Delneste et al., 2003). Another group demonstrated that iDCs growing in the presence of IFN-γ were maturation resistant and they promoted regulatory T cell (Foxp3^{+}) generation (Eljaafari et al., 2009). However, our results indicate that while IFN-γ may be involved in the C1q induced iDC regulatory process, it is not modulated via IFN-γ and/or TNF-α alone. Conceivably, the synergistic effect of IFN-γ and other factors may result in the observed changes in monocyte-to-DC differentiation.

While C1q increased the antigen uptake ability and secretion of selective cytokines by iDCs, allostimulation by C1q treated iDCs was analogous to that of control iDCs. This observation was intriguing, since our results so far indicated that C1q treatment modulates DC differentiation and induces the development of iDCs with monocyte-macrophage-like (non-APC-like) characteristics. In particular, the reduction of CD86 in response to C1q suggested that C1q treated iDCs might have decreased influence on T cell priming. In contrast, C1q treated iDCs showed similar or slightly

increased T cell stimulation compared to control iDCs. Moreover, similar T cell subsets developed in response to stimulation with C1q treated or untreated iDCs. While CD86 is an important co-stimulatory molecule, some of its functions overlap with CD80 (Carreno and Collins, 2002). Furthermore, the influence of alternative costimulatory mechanisms, such as the OX40/OX40-ligand pathway (Redmond et al., 2009) cannot be discounted. Additionally, our previous results revealed that C1q treated iDCs secrete high amounts of IFN-γ. Since DCs highly influence T_H1/T_H2 -cell differentiation, it seems probable that the secretion of IFN-γ at the time of antigen presentation could provide a major mechanism for T cell priming and may compensate for any potential reduction in CD86 signaling.

According to our data, monocyte-DC precursors express high levels of surface C1q during the monocyte-to-DC transition (day 1-2) until firm commitment to the DC lineage is achieved (day 3), while secreted C1q levels remain steady on days 1-4. Previous studies have shown that macrophages and maturing dendritic cells, but not monocytes, synthesize and express C1q (Kaul and Loos, 2001; Vegh et al., 2003). However, our results reveal that fresh monocytes carry C1q on their surface even at day 0, when they have not been exposed to DC growth factors. Furthermore, our experiments indicate that monocytes retain an intracellular pool of C1q. Monocytes may react to injurious stimuli by translocation of this intracellular C1q pool to the cell surface owing to their extraordinary sensitivity to danger signals. Alternatively, the presence of surface C1q may indicate that monocytes capture circulating C1q through C1qRs. In either case,

the functional significance of membrane-associated C1q may represent an as yet unidentified signaling event important in regulating the monocyte-to-DC transition. Our studies show that surface expression of C1q noted on day 0 was maintained beyond day 3 when the precursors were cultured with M-CSF, rather than with DC growth factors (GM-CSF+IL-4). This is consistent with previously published results showing that C1q is present on the surface of macrophages (Kaul and Loos, 2001). The distinct pattern of extracellular C1q expression during the monocyte-to-DC transition (days 3-4) is in agreement with previous observations (including our own unpublished data) demonstrating that C1q is detected on the cell surface and/or in cell free supernatants of iDCs between days 5-7 (Castellano et al., 2007a). Although alternate explanations such as steric hindrance or inaccessibility of the antibody due to the molecular canopy of the cell surface have not been ruled out, our results using C1q domain-specific antibodies suggest that the C1q on the monocyte surface is bound via the globular heads, presumably to gC1qR. In further support, the predominance of gC1qR on fresh monocytes may reflect preferential binding to globular regions of C1q that is free of antigen. Accordingly, C1q globular "heads" would mediate regulatory effects of C1q. In support for this postulate is the fact that the majority of C1q in peripheral blood is associated with the $C1s_2$-$C1r_2$ tetramer forming the pentameric C1 complex. Since the collagen tail of C1q is occupied in this complex, this association only allows the C1q subunit to bind gC1qR and not cC1qR on the cell surface. These observations have led to the suggestion that some or all of the C1q detected on the monocyte surface may be part

of the C1 complex and therefore bound to the cells via gC1qR. Furthermore, cC1qR binds C1q only after it has undergone a conformational change, which may take place after C1q is bound to or released from immune complexes. This therefore indicates gC1qR as the likely candidate to binding monomeric, free C1q or C1 complex on monocytes and iDCs. Cells growing in the presence of monoclonal antibodies specific to gC1qR at the C1q binding site resulted in the development of cells with similar immunophenotypic characteristics to C1q treated cells. These results further suggest that C1q/gC1qR interaction is a requisite of early C1q signaling on these cells, possibly representing a steady state immunoregulatory interaction.

It is well documented that at different stages of development, DCs are specialized in either Ag capture/processing (iDC) or Ag presentation (mature DC). Our studies provide new insight into how a C1q/C1qR system could influence these activities during the critical innate/acquired immunity interface (day 0-3). The release of C1q at this point may act as an autocrine sensor of danger and would reflect the earliest ability of C1q to retrieve extracellular "danger" Ag for re-entry into DCs for processing. Continued release of C1q after the DC lineage is firmly established (day 5-7) correlates with the enhanced ability of iDCs to capture and process Ags in an inflammatory and/or infectious setting. Finally, the lack of C1q and C1qRs on fully mature DCs (Castellano et al., 2004a) is consistent with their advancement into cells specialized in Ag presentation, rather than in Ag capture/processing.

In contrast to C1q released from iDCs, C1q on the monocyte surface would not function to retrieve Ag in the extracellular space. Instead, we propose that this surface C1q reflects an as yet unidentified built-in regulatory mechanism that sustains innate immune functions. Like the suppressive effects of C1q on T cells which act through gC1qR/C1q globular head interactions (Yao et al., 2001a), we hypothesize that the regulatory effects of C1q on monocyte/DC precursors may occur via engagement of globular regions of C1q. Due to the swift nature of the monocyte-to-DC transition, regulatory effects of a C1q/C1qR system would occur within a narrow time frame and would be influenced by the microenvironment (steady state/infection/inflammation).

Our data suggest that the regulatory effects of C1q on DC differentiation and function depend on specific C1q/C1qR interactions. Additionally, cytokines that support innate immune functions (e.g. M-CSF) may also sustain C1q expression on monocytes. The inverse relationship between cC1qR (upregulated) and gC1qR (downregulated) on the cell surface may also reflect preferential signaling through the receptors at distinct stages of DC growth. As outlined in an existing pro-inflammatory induction model, the globular heads of extracellular C1q bind to pathogen associated molecular patterns (PAMPs) or self derived immunogenic material, and thus the collagen tails remain available for stimulating cC1qR on the surface of phagocytic cells. cC1qR may then potentially initiate cell signaling via either CD91 (Paidassi et al., 2008; Trouw et al., 2008) or other cell surface signaling partners. Such antigen bound C1q is likely to be aggregated, which is optimal for induction of the pro-inflammatory response through

cC1qR. A normal response to danger may involve upregulation of cC1qR levels on iDCs to ensure uptake of noxious agents utilizing the Ag-retrieving functions of C1q. The relative lack of cC1qR on fresh monocytes is in agreement with their inferior antigen capturing/processing activity compared to DCs. In the context of inflammatory stimuli, DC maturation would ensue, allowing adaptive immune responses toward the initiating agent. During normal physiology, steady state levels of C1q/C1qR on monocytes would resume once pathogen/danger has been cleared.

Conclusion and Hypothetical Working Model

Our data indicate novel functions for C1q and suggest that the C1q/C1qR system can specifically target cells during early DC differentiation events. The distinct binding orientation of C1q on the cell surface of monocytes and iDCs suggests that specific C1q/C1qR interactions may regulate cells as they transition from the monocyte state (innate immunity) toward the professional APC state (adaptive immunity). Based on these results and observations, we propose that the orientation of C1q - heads versus tails- and the specific receptors engaged control C1q's modulatory effects during DC differentiation. Therefore, in addition to the differential pattern of C1qR expression that we noted during the monocyte-to-DC transition, we speculate that preferential engagement of distinct regions of C1q (globular heads vs. collagen tails) takes place during different stages of DC growth. This putative dual nature of C1q is reflected in conflicting reports of inflammatory induction (Csomor et al., 2007; Gardai et al., 2003), or suppression (Castellano et al., 2007a) of monocytes and DCs by C1q in vitro. Such dichotomy of function would be very similar to the role of the structurally related surfactant protein (Sp)-A and Sp-D in the lung, where they help maintain the steady state environment via binding to the ITIM-containing SIRPα on alveolar cells through their globular head domains. However, when these globular domains interact with PAMPs on foreign organisms, apoptotic cells, or cell debris, the collagenous tails are presented in an

aggregated state to calreticulin and can then initiate ingestion and/or pro-inflammatory responses (Gardai et al., 2003; Pastva et al., 2007).

Based on these data and observations we developed a hypothetical working model. According to our model (Fig. 14), a normal response to "danger" would involve upregulation of cC1qR on iDCs to ensure uptake of noxious agents utilizing the Ag-retrieving functions of C1q. In the context of inflammatory stimuli, DC maturation would ensue, allowing adaptive immune responses toward the initiating agent. During normal physiology, return to steady state levels of C1q/C1qR on monocytes and/or DC precursors would resume once pathogen/danger has been cleared. During steady state, interaction between C1q free of antigenic "cargo" and monocyte-DC precursors does not support DC maturation. We propose that this surface bound C1q reflects a regulatory mechanism to sustain innate immune functions. Similar to the suppressive effects of C1q on T cells which act through gC1qR/C1q interactions, we hypothesize that regulation of monocyte/DC precursors by C1q occurs via engagement of the globular regions. In autoimmune diseases such as SLE, where C1q deficiency is considered to predispose individuals to develop the disease (Ghebrehiwet and Peerschke, 2004b; Walport et al., 1998), a disruption of C1q/gC1qR interactions including those on the monocyte-DC precursor might represent the loss of an important regulatory function. Additionally, in diseases involving C1q/gC1qR mimicry such as HCV infections, escape from adaptive immunity might result from interference with DC differentiation events at the monocyte-to-DC transition.

Taken together, these results suggest that in the absence of danger signals C1q may help maintain steady state conditions by skewing DC differentiation toward cells with monocyte-macrophage-like characteristics. The modulatory effect of C1q during the mono-DC transition may even be more robust than measured by our assays, because secretion and surface expression of C1q by iDCs in culture may already provide C1q related signaling. It is also important to note in this context that our experimental approach represents "over-induced" conditions which are somewhat similar to those employed in gene over-expression assays, which although not physiological, do uncover potential functions of various proteins in their natural context.

While we recognize the important role of C1q in the clearance of apoptotic cells, multiple alternate mechanisms are available for apoptotic uptake by iDCs, including phosphatidylserine recognition, scavenger receptors, and β2-integrins (Maniati et al., 2008). In light of these alternative mechanisms, the loss of C1q's antigen retrieving functions may not be a primary pathogenic event. Instead, a lack of C1q might result in dysregulated progression toward the DC lineage and inappropriate acquired (auto)immunity resulting in autoimmune diseases such as SLE. Because DCs orchestrate a wide range of immune responses, results of our work may, in the long term, contribute to the development of therapeutic modalities aimed at attenuating or even curing a diversity of diseases characterized by either a loss or overabundance of C1q activity and DC abnormalities.
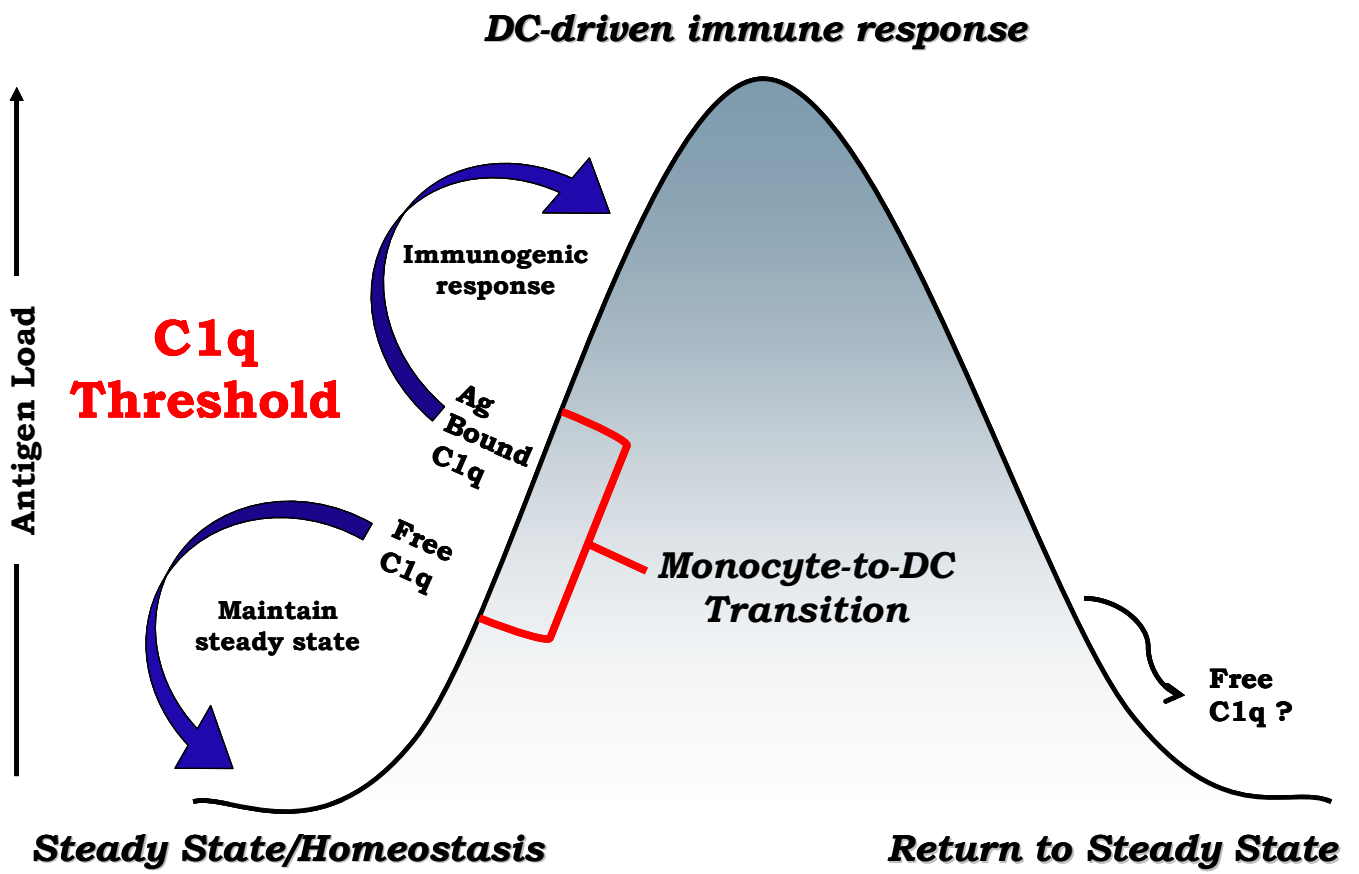
Figure 14. Hypothetical working model.

Future directions

In the future our lab aims to focus in more detail on 1) the regulation of C1q synthesis in monocytes and DCs, 2) the function and maturation of C1q treated DCs, 3) the molecular details of monocyte-to-DC differentiation regulated by C1q, 4) the role of C1q with or without PAMPs or self proteins generated during sterile inflammation, and 5) the role of C1q in T cell differentiation especially with regards to the generation of T regulatory cells.

There is little information about regulatory molecules that may favor or inhibit C1q synthesis. Although macrophages have been shown to display C1q as a type II membrane anchored protein, there is only one recent report that provides evidence that DCs might also express membrane-anchored C1q. Therefore, we aim to uncover whether DCs also carry membrane anchored C1q on their surface. Since membrane C1q is normally tightly bound in the intact membrane through a transmembrane domain located in the A-chain, we will develop an antibody to a peptide derived from the transmembrane domain of the A-chain of C1q for use in flow cytometric and Western blotting studies.

Furthermore, in order to more deeply understand the role of locally synthesized C1q in DC function, our lab aims to establish the mechanism and kinetics of C1q synthesis. To this effect we plan to study the C1q/C1qR expression at the RNA level using RT-PCR, and to determine the number of cells actively producing C1q by ELISpot or real time antigen capture assays. To further elucidate the various factors that influence

C1q/C1qR expression, we will study their synthesis and surface levels under various inflammatory conditions by employing several pro- and anti-inflammatory cytokines and using flow cytometry and RT-PCR.

Thus far we have addressed how C1q treatment regulates mono-DC differentiation and studied the immunophenotype and function of the developing iDCs. We plan to further investigate how different pro- and anti-inflammatory environments influence the maturation and function of C1q treated iDCs. To this end we will culture C1q treated iDCs under various conditions, including with IFN-γ, TNF-α, IL-6, TGF-β, and IL-10, and study the immunophenotype and function (e.g. T cell priming ability) of the developing cells. Similarly, we plan to investigate how currently employed therapeutic agents that influence the inflammatory environment (e.g. TNF-α inhibitors used to treat RA) might regulate C1q/C1qR expression. These results will provide further insight into how C1q may influence the innate/acquired immune interface via regulating DC function.

We also aim to further look into the specific role of IFN-γ in C1q-mediated mono-DC regulation. While our results indicate that IFN-γ alone is not sufficient to modulate mono-DC differentiation in response to C1q, it may still be a necessary and required factor for the process. To this end we will employ IFN-γ inhibitors in order to elucidate whether IFN-γ is wholly or partially responsible for inducing the modulatory effects of C1q. Since the commercially available IFN-γ inhibitors are all monoclonal antibodies, we will first generate $F(ab')_2$ fragments in order to bypass unwanted Fc-receptor mediated

DC activation during the experiment. We are currently exploring the most efficient method for this in our lab.

We are also interested in further dissecting the role of the different functional regions of C1q (gC1q vs. cC1q) during early DC differentiation. Since generating gC1q fragments in an endotoxin-free environment proved to be unproductive, we are currently exploring the use of commercially engineered gC1q peptides. However, while various companies offer custom peptide synthesis and pyrogen free products, the choice of the specific peptide may be challenging due to the unique tertiary structure of the C1q globular heads that are composed of three separate amino acid chains (A, B, and C). We will therefore also employ the globular regions of each chain separately in mono-DC cultures and assess the developing cells. We will use each fragment individually and in combination with each other. The possible pitfall of these experiments is that the globular head fragments may not have sufficient conformational similarity to intact C1q; therefore their affinity to gC1qR may be compromised. Globular head fragments generated through collagenase digest of C1q are monomeric, and as such, bind to gC1qR with 50x less affinity than the whole molecule (unpublished data). However, recent preliminary experiments in our lab indicate that despite the reduced affinity, 2-6x molar excess of globular head fragments are sufficient to regulate DC differentiation (data not shown). Therefore we are confident that the gC1q fragments of each chain will also interact with gC1qR either individually or in combination with each other.

Lastly, we are actively investigating the molecular details of extracellular and intracellular mono-DC signaling events in response to C1q. The C1q receptors—gC1qR and cC1qR—are unconventional, non-transmembrane surface proteins, which signal by proxy. However, they make up for their lack of a direct conduit into intracellular elements of the cell by forming docking/signaling complexes with transmembrane partners. Because of the diversity of biological responses mediated by C1q receptors, the partner molecules are predicted to be different, depending on the cell type and the cellular response. Some studies have indicated that the partner for cC1qR/CR (calreticulin) on macrophages is CD91. The difference in the distribution of cC1qR and CD91 on the cell surface during the monocyte-to-DC transition suggests however, the existence of distinct signaling pathways involving other receptor partners (Fig. 11A-F). In support of this premise is the demonstration that cC1qR mediated cellular trafficking is not CD91 dependent, indicating the usage of alternative trafficking pathways. As a possible alternative signaling partner, scavenger receptor class-A (SR-A), a prominent scavenger receptor of macrophages and dendritic cells, was shown to serve as a primary cell surface molecule for the recognition and internalization of calreticulin. While the signaling partner for gC1qR on endothelial cells has been identified to be a member of the β1-integrin (CD29) and possibly CD44, not much is known about the molecule(s) on DCs that gC1qR may partner with. Our preliminary experiments (data not shown) also indicate that DC-SIGN may bind to cC1qR and gC1qR. Therefore, identifying the signaling partners for gC1qR and cC1qR on DCs will be important if we are to define the

C1qR-mediated intracellular signaling pathways on DCs. In these experiments potential signaling partners for C1qR will be identified by antigen capture ELISA, Western blotting and fluorescence microscopy and activation of various signaling mediators will be tested using flow cytometry based cell phosphorylation assays and antigen capture ELISA.

__Long Term Goals__

The present work was primarily undertaken to procure molecular insight into the age-old question: *how does deficiency in C1q predispose an individual to develop autoimmune diseases such as SLE?* While the work presented here corroborates the hypothesis that in addition to its significant role in the removal of self-waste, C1q functions as an important molecular switch that regulates the monocyte to dendritic cell transition, our long-term goal is to define the precise molecular mechanism(s) underlying the *loss of peripheral tolerance* in the absence of C1q. Previous studies performed in our laboratory (Chen et al., 1994) and corroborated by others (Kittlesen et al., 2000) have shown that engagement of C1q with its cognate T cell receptors induces a suppressive effect on the same T cells – a fact that is often exploited by microbial pathogens including HCV. Whether this constitutes a critical function for terminating T cell responses is not clear and remains to be examined. Another critical question, to be examined is the emerging role of C1q in modulating or generating the immunosuppressive Foxp3$^+$ regulatory cells in peripheral lymphoid tissues. Although these cells are primarily generated in the thymus where they function to suppress T cell effector responses to self antigens, there is evidence to suggest that they are also generated in peripheral lymphoid tissues. Therefore dissection of the role of locally produced C1q with gC1qR and/or cC1qR on cognate DCs and their T cell partners would be significant. Finally, elucidation of the role of macromolecular C1, which is synthesized by monocytes and DCs – together with its subcomponents C1r and

C1s as well as its regulator, C1 inhibitor – would be significant as it intimates that C1 inhibitor closely monitors the role of C1 in a sterile or infectious inflammation. The presence of C1 inhibitor may thus regulate how C1q, which binds to a plethora of microbial or non-microbial activators at inflammatory sites, can communicate with DCs or T cells to enhance or abort immune responses.

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