STATE-OF-THE-ART RESEARCH ON C1Q AND THE CLASSICAL COMPLEMENT PATHWAY

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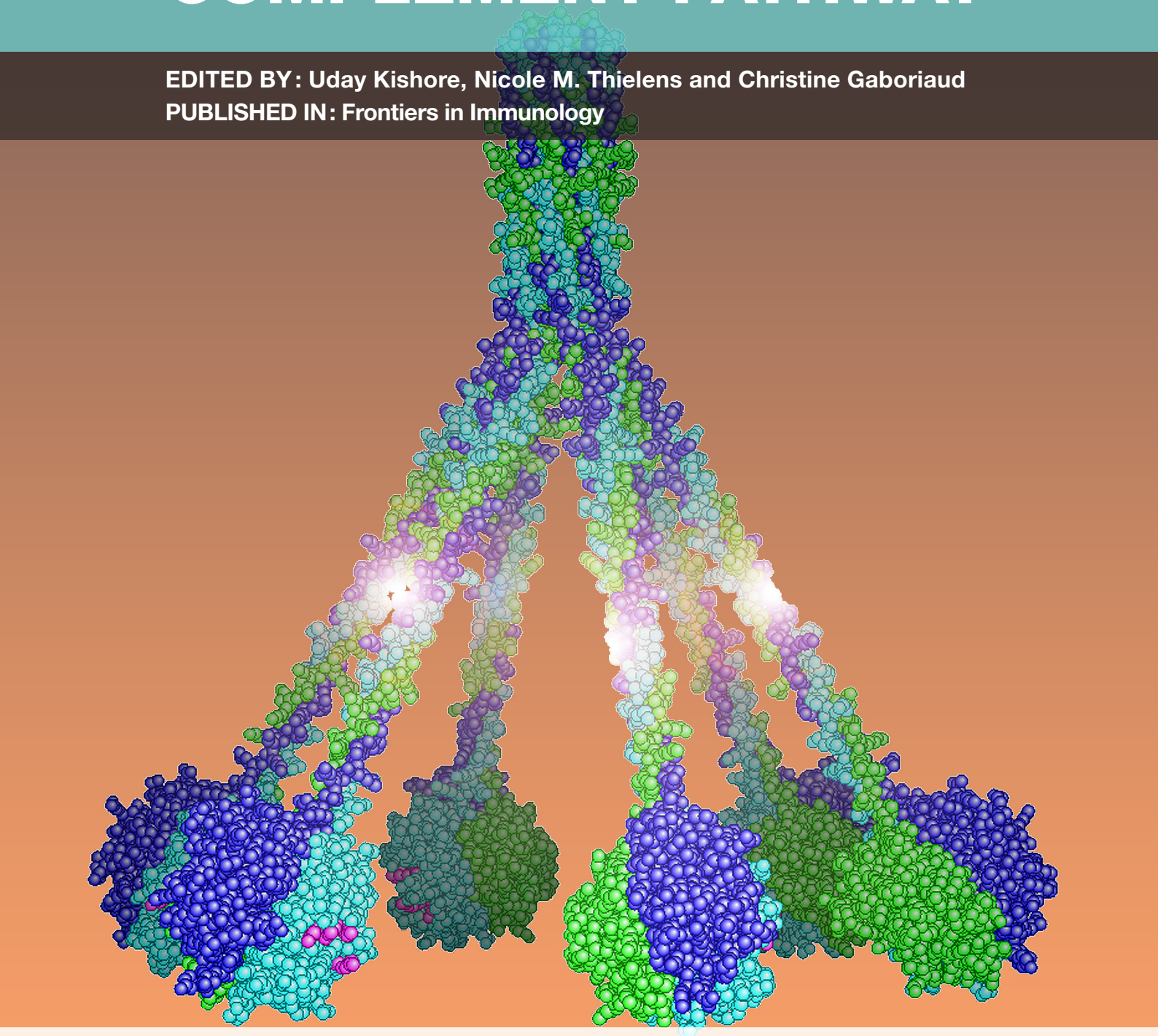

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STATE-OF-THE-ART RESEARCH ON C1Q AND THE CLASSICAL COMPLEMENT PATHWAY

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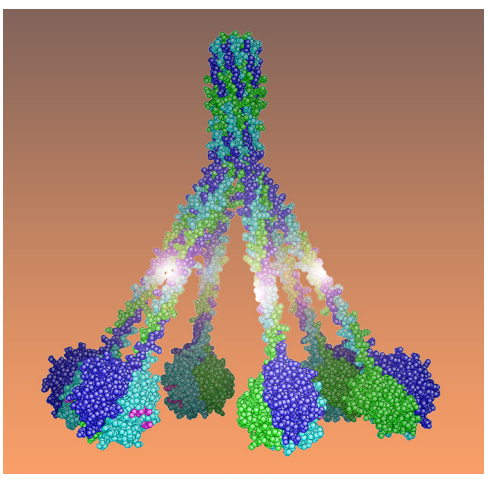

Model of the human C1q molecule, illustrating its multi-chain organization. It is a surface pattern recognition protein including 6 chains of C1qA (colored in dark blue), 6 of C1qB (in green), 6 of C1qC (in cyan).

The structure of the heterotrimeric recognition globular domain has been solved experimentally by X-ray crystallography. The other collagen-like parts of the molecule have been modelled.

White colour at the middle of the collagen-like stems highlights the approximate location of the effector proteases binding site; magenta spots locate a recognition binding site.

Figure by Christine Gaboriaud

C1q is the target recognition protein of the classical complement pathway and a major connecting link between innate and acquired immunity. As a charge pattern recognition molecule of innate immunity, C1q can engage a broad range of ligands derived from self, non-self and altered self via its heterotrimeric globular (gC1q) domain and thus trigger the classical complement pathway. The trimeric gC1q signature domain has been identified in a variety of non-complement proteins that can be grouped together as a C1q family. C1q circulates in serum as part of the C1 complex, in association with a catalytic tetrameric assembly of two homologous yet distinct serine proteases, C1r and C1s. Binding of C1q to appropriate targets leads to sequential activation of C1r and C1s, the latter being able to cleave complement components C4 and C2 thereby triggering the complement cascade. Activation of the classical pathway plays an important role in innate immune protection against pathogens and damaged elements from self. However, its involvement has been shown in various pathologies including ischemia-reperfusion injury and hereditary angioedema. Unexpected roles for the classical pathway have also been discovered recently, linked to both physiological and pathological aspects of development, including brain and

cancer cells. These new perspectives should arouse renewed interest in a search for specific inhibitors of the classical pathway. In addition, C1q has recently been shown to have a number of functions that are independent of the activation of the classical pathway.

This research topic is aimed at providing a state-of-the-art overview of the classical pathway, including, but not restricted to emerging functions of C1q and of the C1 complex, as well as pathological consequences of C1 activation or of the presence of anti-C1q autoantibodies. Contributions are included in the areas such as structural basis of C1q ligand recognition, C1q family proteins, inhibitors of the classical pathway identified in pathogens and improved derived inhibitors, structural determinants of the substrate specificities of C1r and C1s, elucidation of the architecture of C1, structural and functional homology of C1 with the initiating complexes of the lectin complement pathway, and novel involvement of C1q in processes such as ageing, cancer, synaptic pruning, and pregnancy.

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EDITORIAL
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Editorial: State-of-the-Art Research on C1q and the Classical Complement Pathway

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Keywords: complement, C1 complex, C1q, gC1q structure, C1s, classical pathway activation, autoimmunity, emerging functions

The Editorial on the Research Topic

State-of-the-Art Research on C1q and the Classical Complement Pathway

Complement protein C1q is a fascinating innate immune molecule. C1q is the first subcomponent of the classical complement pathway. Its primary three-chain structure (A, B, and C chains), which is composed of a triple-helical collagen-like region and a C-terminal ligand-recognizing globular head (gC1q) domain, yields a tulip-like organization with six gC1q domains, each representing a heterotrimer (the C-terminal regions of the A, B, and C chains) (1). In addition to binding to immune complexes containing IgG and IgM, the gC1q domain also engages with a number of self and non-self ligands. A summary of the structural basis of C1q–ligand interactions has been elegantly presented in the review by Gaboriaud et al. For example, it shows that C1q can recognize multiple ligands on the apoptotic cell surface, which illustrates its versatile surface recognition properties. A common binding area for several of these non-immune ligands has been observed in the subunit C, which could play a role in restricting the activation of the classical complement pathway.

It has become evident over the course of the last two decades that the gC1q domain is widely conserved across a diverse range of vertebrate and invertebrate proteins (2). The functions of these proteins can range from being immunological to structural. Through a review article, Ghebrehiwet et al. have cited two examples of C1q functions that are distinct from its involvement in the classical pathway: its ability to induce apoptosis in prostate cancer cells, and to modulate vascularization for fetal–maternal interaction. Given the existence of a C1q–TNF superfamily based on the remarkable structural similarities between C1q and TNF family members (1), a good argument has been made for a cytokine-like property of C1q. The complexity and diversity related to the functions of C1q family have been greatly exemplified by a structure–function review article by Colombatti et al., who describe the functions associated with the gC1q domain of two C1q family members – such as EMILIN and multimerin. A slight structural variation from the prototypical structure, as revealed by the NMR solution structure, is insightful, purporting one residue in the interaction of gC1q domain with $\alpha_4\beta_1$ and $\alpha_9\beta_1$ integrins.

In recent years, a number of functions of C1q have emerged that are complement independent. This is reflected in the local synthesis of C1q by various immune and non-immune cells. The diversity of C1q functions includes its involvement in dendritic cell maturation, immune modulation, cell differentiation, cancer progression, neuronal synapse pruning, and pregnancy. This has been extensively summarized by Kouser et al. In the backdrop of a recent observation that C1q gene knockout mice show nearly all features of preeclampsia, the role of C1q in normal and complicated pregnancies

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has become a burning issue in the reproductive immunology. In this issue, Madhukaran et al. have reported a link between the expression of the transcription factor PU.1 and C1q in human trophoblasts and stromal cells, similar to DCs and macrophages, using early decidual tissue.

Given its involvement in a wide range of homeostatic functions, it is not surprising that C1q is central to many human diseases. C1q, as a key molecule in self-tolerance mechanisms, is involved in clearing immune complexes and apoptotic/necrotic cells. C1q-deficient mice have been shown to have lupus-like symptoms. However, this issue is further confounded by the fact that anti-C1q autoantibodies are found in a number of pathological situations, more so in systemic lupus erythematosus (SLE). These autoantibodies are certainly required for the development of lupus nephritis. Since C1q itself is an IgG-binding protein, identification and characterization of anti-C1q autoantibodies is a challenging task. Mahler et al. have addressed the technicalities of this endeavor, while reassessing the pathological consequences of such autoantibodies in a disease context. In line with the involvement of C1q in tolerance and SLE, Ghebrehiwet et al. have addressed the importance of monocyte surface expressed C1q in association with C1r and C1s. This review examines the role of cell-bound C1q in capturing and processing circulating immune complexes and pathogen-associated molecular patterns (PAMPs). The ability of C1q to modulate PAMP-recognizing receptors (PRRs) makes it a potent and versatile immune surveillance molecule of the innate immunity.

The final section of the issue is dedicated to the understanding of the mechanisms underlying activation of the classical pathway and their specific inhibition for therapeutic purposes.

In an original research article, Wijeyewickrema et al. aim at further deciphering the interaction of the C1s protease with its C4 protein substrate. Previous studies had provided evidence for homologous C4 interaction exosites in the CCP and serine protease domains of both C1s and MASP-2 (3). The authors focus here on the role of C1s Lys628 residue of the serine protease domain. Using site-directed mutagenesis and a peptide substrate library, they show that this residue plays a different role in cleaving peptide versus protein substrates by interacting with C4 in order to facilitate its cleavage. The architecture of the active site at this position is markedly different in C1s compared with MASP-2 (4), which might provide clues toward designing specific inhibitors of the classical pathway.

In a review article, Sharp et al. focus on complement inhibitors of the classical pathway for application in transfusion medicine. Uncontrolled antibody-initiated complement activation plays a central role in hemolytic diseases such as acute intravascular hemolytic transfusion reaction (AIHTR). The authors have identified peptide inhibitors of C1 (PIC1) derived from a region of the coat protein of astrovirus with homology to human neutrophil peptide 1 that inhibits complement activation by binding to the collagen-like regions of C1q (5). They have developed a simple rat model of AIHTR for future preclinical studies of PIC1. A review of complement inhibitors, marketed as well as currently under development, is presented, and their therapeutic potential in transfusion and blood disorders is discussed.

In their perspective article, Gaboriaud et al. provide an update on the current knowledge about the structural basis of the mechanisms involved in the assembly and activation of the C1 complex. The main protein players involved in C1 activation and its control are presented together with the molecular dissection strategy used to define structure–function relationships of C1 subcomponents and to decipher key protein–protein interactions. However, the conformational changes required for allowing C1 activation following target binding are still not elucidated, and this is one among 16 still unanswered questions identified by the authors. Hopefully, new technological developments in structural biology, such as a combination of X-ray crystallography and electron microscopy, will help fulfilling this "mission impossible."

This special topic issues clearly highlights the diversity and complexity associated with structures and functions of C1q and C1q family members. We have come a long way in understanding the structural basis of the gC1q interaction with self and non-self ligands. While additional members of C1q family are still being identified and characterized, the importance of the gC1q domain in the evolutionary history of animals is unique. From being a prototypical innate immune molecule, C1q is now regarded as an independent modulator of a diverse range of functions that are not dependent on its involvement in the complement activation. These complement-independent functions of C1q are likely to be the major thrust of research in coming years.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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The human C1q globular domain: structure and recognition of non-immune self ligands

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C1q, the ligand-binding unit of the C1 complex of complement, is a pattern recognition molecule with the unique ability to sense an amazing variety of targets, including a number of altered structures from self, such as apoptotic cells. The three-dimensional structure of its C-terminal globular domain, responsible for its recognition function, has been solved by X-ray crystallography, revealing a tightly packed heterotrimeric assembly with marked differences in the surface patterns of the subunits, and yielding insights into its versatile binding properties. In conjunction with other approaches, this same technique has been used recently to decipher the mechanisms that allow this domain to interact with various non-immune self ligands, including molecules known to provide eat-me signals on apoptotic cells, such as phosphatidylserine and DNA. These investigations provide evidence for a common binding area for these ligands located in subunit C of the C1q globular domain, and suggest that ligand recognition through this area down-regulates C1 activation, hence contributing to the control of the inflammatory reaction. The purpose of this article is to give an overview of these advances which represent a first step toward understanding the recognition mechanisms of C1q and their biological implications.

Keywords: C1q, complement, innate immunity, ligand recognition, X-ray crystallography

INTRODUCTION

Triggering of the classical complement pathway is mediated by C1, a multimolecular complex resulting from the association of a recognition subunit, C1q, and a Ca^{2+}-dependent tetramer comprising two copies of two serine proteases, C1r, and C1s (Cooper, 1985; Gaboriaud et al., 2004). C1q is a 460-kDa hexameric protein assembled from six heterotrimeric collagen-like fibers, each being prolonged by a C-terminal globular domain which mediates the recognition function of C1 (Gaboriaud et al., 2003, 2004; Kishore et al., 2004). A major characteristic of this domain lies in its ability to sense and engage an amazing variety of ligands (Cooper, 1985; Kishore et al., 2004). Thus, C1q is classically known for its ability to bind IgG- and IgM-containing immune complexes. In addition, it recognizes the lectin SIGN-R1, C-reactive protein, and other pentraxins bound to pathogens and other surfaces, as well as various molecular motifs on several Gram-negative bacteria and viruses (Cooper, 1985; Szalai et al., 1999; Thielens et al., 2002; Kishore et al., 2004; Kang et al., 2006). In most cases, recognition of these non-self ligands by C1q triggers activation of the classical complement pathway, thereby contributing to their elimination through enhanced phagocytosis, lysis, and inflammation.

This traditional view of the biological role of C1q should be reconsidered in light of recent studies providing evidence that C1q has the ability to sense many altered structures from self, including the pathological form of the prion protein (Klein et al., 2001; Erlich et al., 2010), β-amyloid fibrils (Tacnet-Delorme et al., 2001), modified forms of low-density lipoprotein (Biro et al., 2007, 2010), and apoptotic cells (Taylor et al., 2000; Navratil et al., 2001). Recognition by C1q triggers efficient clearance of apoptotic cells by phagocytes, but in this case lysis and inflammation are both inhibited, thus contributing to the maintenance of immune tolerance (Nauta et al., 2004; Fraser et al., 2009). Recent investigations on the C1q targets at the apoptotic cell surface have revealed that three molecules known to provide "eat-me" signals, phosphatidylserine, DNA, and calreticulin, are recognized by C1q, suggesting a multiligand-binding process (Païdassi et al., 2008a,b, 2011).

The three-dimensional (3-D) structure of the heterotrimeric globular domain of C1q, responsible for the recognition function of this protein, has been solved by X-ray crystallography, giving insights into its versatile binding properties (Gaboriaud et al., 2003). The same technique, as well as other approaches, have been recently applied to decipher the interaction of this domain with various non-immune self ligands, including molecules acting as eat-me signals on apoptotic cells, providing evidence for a common binding area (Païdassi et al., 2008a; Garlatti et al., 2010). The purpose of this article is to give an overview of these advances which shed light on the recognition of self ligands by C1q and reveal possible implications in the regulation of C1 activation.

ARCHITECTURE OF THE HUMAN C1q GLOBULAR DOMAIN

The 3-D structure of the C-terminal globular domain of human C1q, obtained after digestion of the collagenous moiety of the protein with collagenase, has been solved by X-ray crystallography to a resolution of 1.9 Å (Gaboriaud et al., 2003). The structure revealed a globular, almost spherical heterotrimeric assembly, with a diameter of about 50 Å, the N- and C-termini of each subunit emerging at the base of the trimer, in close vicinity to one another (**Figures 1A,B**). A major information from the structure

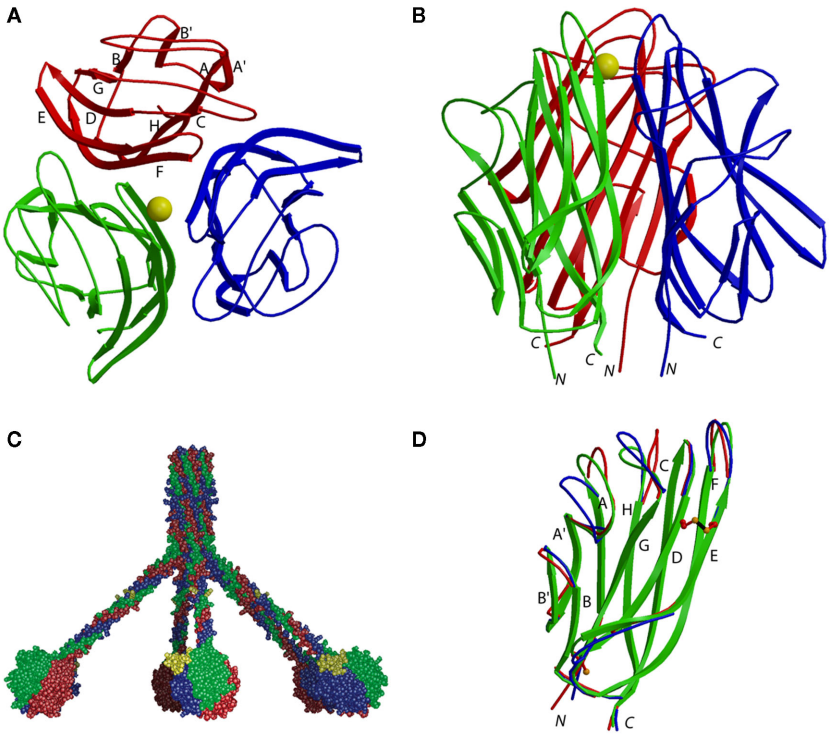

FIGURE 1 | Structure of the C1q globular domain. (A) Top view and **(B)** side view of the heterotrimeric assembly. Subunits A, B, and C are shown in blue, green, and red, respectively. β-strands are labeled according to the tumor necrosis factor nomenclature, and the Ca^{2+} ion is represented as a golden sphere. N and C indicate the N- and C-terminal ends of each gC1q subunit. **(C)** Side view of the three-dimensional C1q model derived from the structure of the globular domain. The N-linked oligosaccharide attached to each globular domain is represented in yellow. **(D)** Structure of the gC1q subunits. Subunits A, B, and C are superimposed. Only the free cysteine and disulfide bond of subunit C are displayed for clarity. (Modified from Gaboriaud et al., 2003, 2004).

was that the gC1q subunits are arranged clockwise in the order A, B, C when the assembly is viewed from the top, this indication enabling us to derive a 3-D model of the collagen-like triple helix of C1q, and thereby to reconstruct the whole C1q molecule (Gaboriaud et al., 2003, 2004). In the resulting C1q model (**Figure 1C**), the B subunit of each globular domain lies on the outer part of the molecule, whereas A and C are positioned inside. As discussed later, it is very likely that this particular configuration has direct implications in terms of ligand recognition and C1 activation.

The three subunits each exhibit a 10-stranded β sandwich fold with a jelly roll topology homologous to the one described initially for tumor necrosis factor (Eck and Sprang, 1989; Jones et al., 1989) and subsequently for members of the gC1q family (Shapiro and Scherer, 1998; Bogin et al., 2002; Kvansakul et al., 2002), consisting of two five-stranded β-sheets (A′, A, H, C, F and B′, B, G, D, E), each made of anti-parallel strands (**Figure 1D**). Comparison of the three subunits indicates strong conservation of the β-strands and significant variability in the loops connecting them, a characteristic that also applies to other gC1q domains. In addition to a free cysteine conserved in all gC1q domains, which is essentially buried in the structure, each subunit of human C1q contains two other cysteines engaged in a disulfide bond (**Figure 1D**).

Assembly of the C1q heterotrimer involves a tight association of the subunits, as shown by a total buried surface of 5490 $Å^2$ equally contributed by each subunit, this value being significantly less, however, than those in collagens VIII and X (Bogin et al., 2002). Trimerization involves a central interface as well as lateral interactions which in both cases are hydrophobic at the base of the trimer and become more polar when going to the apex. This results in the formation of a discontinuous central channel which is closed at both extremities. Interestingly, despite its heterotrimeric structure, the C1q globular domain assembles in the same way as its homotrimeric counterparts in ACRP30 and collagens VIII and X. Nevertheless, attempts to assemble gC1q subunits as homotrimers *in silico* were found to result in severe steric clashes, particularly at the level of lateral interactions, thus providing a structural basis for their natural propensity to associate only as heterotrimers (Gaboriaud et al., 2003). The structure of the C1q globular domain also reveals the presence of a Ca^{2+} ion bound at the top of the assembly (**Figures 1A,B**). The binding site is asymmetrical relative to the trimer, considering that Ca^{2+} is coordinated by oxygen ligands contributed by subunits A and B, but is not connected to subunit C. In contrast to the buried Ca^{2+} cluster observed in collagen X (Bogin et al., 2002), the single Ca^{2+} ion of C1q is exposed to the solvent and defines the upper end of the central channel. In addition to contributing to the stability of the heterotrimeric

assembly, the Ca^{2+} ion may have a functional role. Thus, the loss of Ca^{2+} has been postulated to modify the direction of the electric moment of the C1q globular domain and thereby to influence the recognition of immune targets such as C-reactive protein and IgG (Roumenina et al., 2005). The fact that the Ca^{2+} ion is accessible to the solvent also opens the possibility of a direct implication in the recognition of certain charged targets (Gaboriaud et al., 2003).

THE HETEROTRIMERIC STRUCTURE OF THE C1q GLOBULAR DOMAIN AS THE KEY TO ITS BINDING VERSATILITY

A striking feature of the structure of the C1q globular domain lies in the fact that the three subunits exhibit marked differences in their surface patterns, with respect to both charged and hydrophobic residues (**Figure 2**). Thus, subunit A mainly shows a combination of arginine and acidic residues scattered on its surface (**Figure 2A**). Subunit C also shows a combination of basic and acidic residues spread over the surface (**Figure 2C**). In contrast, positively charged residues are predominant on the surface of module B, with in particular a cluster of three arginines ArgB101, ArgB114, and ArgB129. The latter two residues, which have been proposed to be involved in the interaction with IgG (Marqués et al., 1993), markedly protrude outside the structure (**Figure 2B**). Several hydrophobic residues are exposed to the solvent on the external face of each subunit, the most striking example being the IleB103, ValB105, ProB106 cluster lying over the ArgB101, ArgB114, ArgB129 triad (**Figure 2B**). In contrast, the only accessible aromatic residues (TyrC155, TrpC190) are found on the equatorial area of subunit C (**Figure 2C**). The top of the heterotrimer (**Figure 2D**) shows a predominance of positive charges mainly contributed by lysine residues. In each subunit, several hydrophobic patches and aromatic residues are also exposed.

The heterotrimeric structure of the C1q globular domain is very likely a major determinant of its versatile recognition properties. Thus, because they display quite different surface patterns in terms of charged and hydrophobic residues, the three subunits are expected to mediate different individual binding properties. In addition, considering the tightly packed structure of the domain, it appears likely that recognition of certain ligands will involve residues contributed by several subunits, thereby considerably enlarging the spectrum of the C1q targets. This assumption appears consistent with the observation that many C1q ligands have been found to interact in a significant way with different subunits of its globular domain (Kishore et al., 2003). As discussed later, the diversity of the recognition modes of C1q is well illustrated by the models proposed for C-reactive protein and IgG1, where binding is thought to involve the apex of the C1q globular domain, and the equatorial region of subunit B, respectively (Gaboriaud et al., 2003).

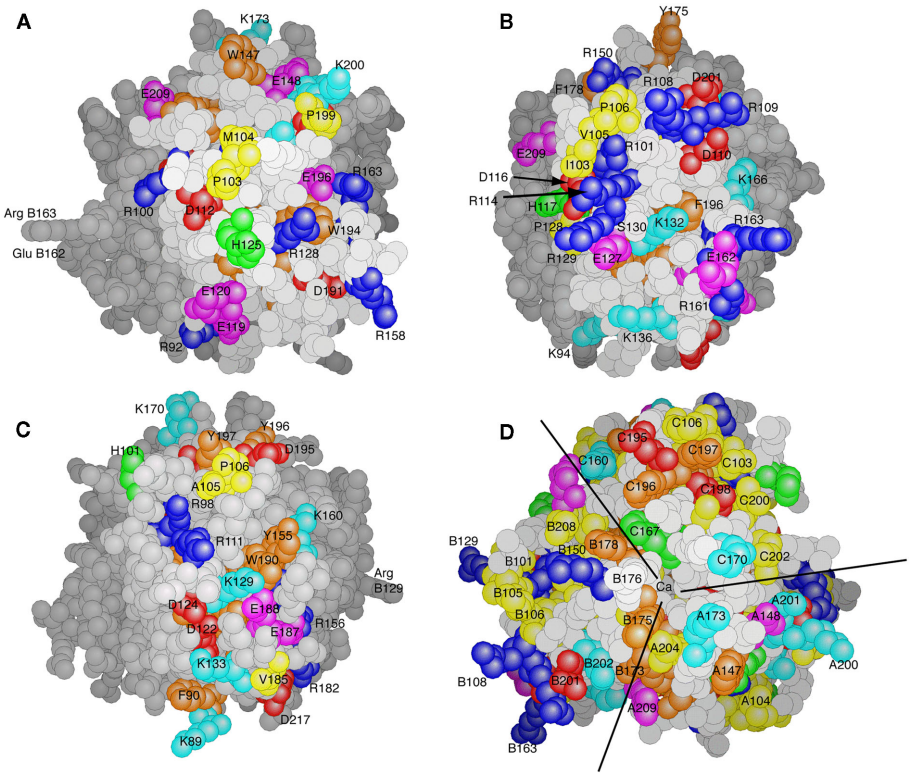

FIGURE 2 | Surface properties of the C1q globular domain. **(A–C)** Side views of the heterotrimer seen from subunits A, B, and C, respectively. **(D)** Top view of the heterotrimer. The side chains of Arg, Lys, His, Asp, and Glu residues are shown in deep blue, light blue, green, red, and magenta, respectively. Hydrophobic residues are shown in yellow, and aromatic ones are in orange. The lines in **(D)** indicate the approximate subunit boundaries. (From Gaboriaud et al., 2003).

C1q RECOGNIZES MULTIPLE LIGANDS AT THE APOPTOTIC CELL SURFACE

Recent investigations on the targets recognized by C1q at the apoptotic cell surface have allowed us to identify several ligands (Païdassi et al., 2008a,b, 2011). In all cases, the C1q moiety responsible for binding has been shown to be the globular domain.

Phosphatidylserine is currently one of the best documented recognition signal required for apoptotic cell clearance. Its tethering is mediated directly by phagocyte receptors, with or without the help of soluble bridging molecules (Savill et al., 2002). We have recently shown that C1q is one of the molecules that bridge phosphatidylserine exposed at the surface of early apoptotic cells to the phagocyte (Païdassi et al., 2008a). The interaction is mediated by its globular domain, which recognizes the phosphoserine moiety of phosphatidylserine. Annexin V, which binds avidly to phosphatidylserine, inhibits the interaction, and C1q and its globular domain both co-localize with phosphatidylserine on the apoptotic cell surface. Surface plasmon resonance analysis of the interaction between the C1q globular domain and phosphatidylserine has revealed high-affinity (**Figure 3A**) and the phosphoserine binding site on C1q has been identified by X-ray crystallography (**Figure 4**).

Nuclear splitting and DNA fragmentation are well-established features of apoptosis, and the generation of auto-antibodies directed against chromatin components is a characteristic of autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (Shoenfeld et al., 1987). It was recently demonstrated that nucleic acids are rapidly exposed during apoptosis and provide ligands for C1q binding (Elward et al., 2005). In an attempt to characterize C1q binding to DNA, we have shown for the first time that the globular region of C1q displays a lectin-like activity that contributes to DNA binding (Païdassi et al., 2008b). This observation is consistent with the observation that treatment of apoptotic cells with DNase decreases significantly binding to C1q. Studies at the molecular level have demonstrated that the C1q globular domain binds DNA through its pentose moiety, and that deoxy-D-ribose inhibits formation of complexes between DNA and the C1q globular domain (**Figure 3B**). The structure of the deoxy-D-ribose binding site on the C1q globular domain has been deciphered at high resolution by X-ray crystallography.

Calreticulin was first identified as a receptor for the collagenous moiety of C1q and the collectins at the surface of phagocytes. However, based on more recent studies, it is now widely accepted that calreticulin is a major recognition signal at the apoptotic cell surface, acting as an "eat-me" signal in the uptake of apoptotic cells by macrophages (Gardai et al., 2006; Obeid et al., 2007). This latter finding evokes a double play for this protein, as both a phagocyte receptor and an "eat-me" signal on the apoptotic cell surface, with the help of the bridging molecule C1q. In support of this hypothesis, we have recently demonstrated that the C1q globular domain, responsible for C1q binding to apoptotic cells, binds calreticulin with high-affinity and co-localizes with this protein on the apoptotic cell surface (**Figure 3C**; Païdassi et al., 2011).

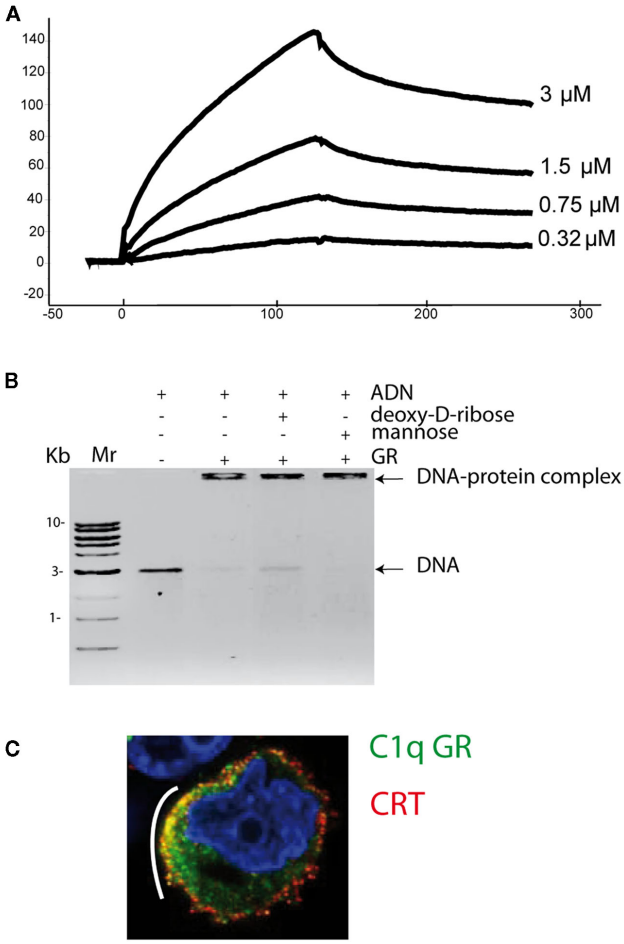

FIGURE 3 | C1q binding to apoptotic cell ligands. (A) Surface plasmon resonance analysis of the binding of the C1q globular domain to immobilized phosphatidylserine. The concentrations of the C1q globular domain are indicated. **(B)** Electrophoretic mobility shift assay showing that complex formation between DNA and the C1q globular domain is partly inhibited by 100 mM deoxy-D-ribose. Mannose was used as a negative control. DNA molecular weight markers are shown on the left. **(C)** Early apoptotic HeLa cells were submitted to a double-immunofluorescence labeling for the C1q globular domain (C1q GR, green) and calreticulin (CRT, red) followed by confocal laser microscopy detection. Nuclei were labeled with Hoechst (blue). The white bow indicates areas where the C1q globular domain co-localizes with calreticulin. (From Païdassi et al., 2008a,b, 2011).

A COMMON BINDING AREA FOR NON-IMMUNE SELF LIGANDS

To decipher the recognition properties of the C1q globular domain, its X-ray structure has been solved in the presence of three different small ligands corresponding to molecular determinants recognized by C1q in larger target molecules from self: (i) phosphoserine, the motif recognized in phosphatidylserine, (ii) deoxy-D-ribose, a specific determinant of DNA, and (iii) two repetitive units of heparan sulfate (Païdassi et al., 2008a; Garlatti et al., 2010). The three structures were solved using crystal soaking because attempts to generate co-crystals of the C1q globular domain in complex with a ligand were unsuccessful. The soaking technique introduces space constraints, and is more favorable to small ligands. The smallest ligand tested, deoxy-D-ribose, was observed at the highest resolution (1.25 Å) in a convincing binding site located in subunit C of the C1q globular

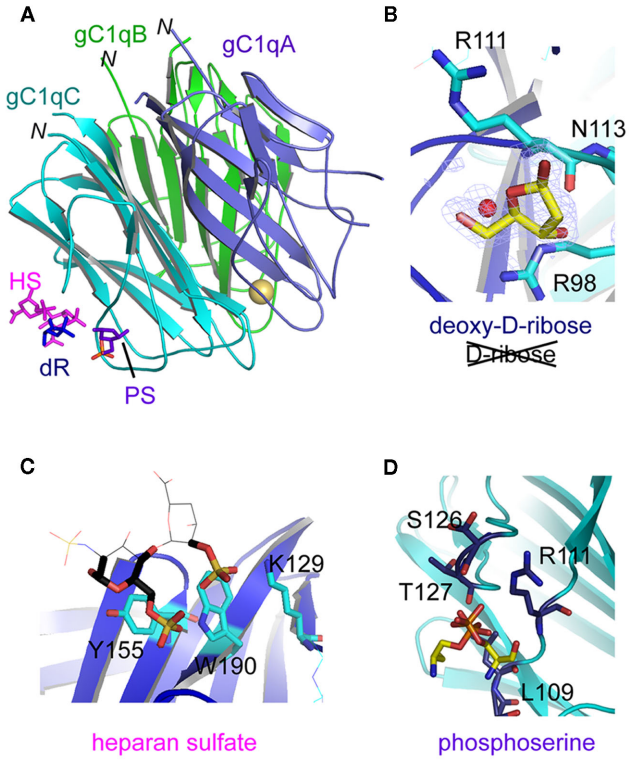

FIGURE 4 | Location of three non-immune self ligand-binding sites in the C1q globular domain. (A) Overall view of the C1q globular domain indicating the relative positioning of the binding sites for heparan sulfate (HS), deoxy-D-ribose (dR), and phosphoserine (PS). **(B–D)** Detailed views of the binding sites for deoxy-D-ribose, heparan sulfate, and phosphoserine, respectively. Residues of the C subunit involved in the interaction are shown. In **(C)**, the ligand portion displayed in sticks corresponds to the major interpretable extra electron-density. In (**D**), a double conformation was used to interpret the extra-density corresponding to phosphoserine. (Modified from Garlatti et al., 2010 and Païdassi et al., 2008a).

domain (Garlatti et al., 2010). It is accommodated in a small pocket lying between ArgC98 and ArgC111 (**Figure 4B**). Two of its hydroxyl groups are stabilized by direct and water-mediated polar interactions with AsnC113, ArgC111, and ArgC118. The interaction is clearly specific to deoxy-D-ribose, since no ligand fixation could be detected when the same protocol was applied using D-ribose. Indeed, the additional hydroxyl group present in ribose would clash sterically with AsnC113 in the observed configuration, providing a structural basis for the strict specificity of C1q toward deoxy-D-ribose. The other two ligands investigated, phosphoserine and heparan sulfate, are both negatively charged, and interact approximately in the same area of the C subunit of the C1q globular domain (**Figure 4A**), although they differ in their precise interaction modes (Païdassi et al., 2008a; Garlatti et al., 2010). Thus, heparan sulfate is interacting with TyrC155, TrpC190, and LysC129 (**Figure 4C**), whereas phosphoserine binds ArgC111, SerC126, and ThrC127 (**Figure 4D**). The sulfate/phosphate binding propensity of ArgC98, ArgC111, TyrC155, and TrpC190 suggests that this area has the ability to bind larger polyanionic molecules. If this hypothesis is correct, then the larger polyanionic molecules DNA and heparin are expected to compete with each other because of the close proximity of their binding sites. This hypothesis has been tested using competition experiments, providing evidence that, indeed, DNA and heparan sulfate fragments each inhibit in a dose-dependent manner C1q binding to a HS-coated surface, in complete agreement with X-ray crystallography analyses (Garlatti et al., 2010).

THE LOCATION OF A LIGAND-BINDING SITE ON THE C1q GLOBULAR DOMAIN CORRELATES WITH ITS C1 ACTIVATION POTENTIAL

According to the C1q model derived from the X-ray structure of its globular domain (**Figure 1C**), the binding sites for phosphoserine, deoxy-D-ribose, and heparan sulfate, all located in subunit C, would be positioned on the inner face of the C1q cone, and oriented toward the target (**Figures 4A** and **5A**). In contrast, the IgG binding site has been proposed to lie on the equatorial region of subunit B, on the outer part of the C1q molecule (Gaboriaud et al., 2003; Garlatti et al., 2010). In light of our current knowledge of the structure and activation mechanism of the C1 complex (Budayova-Spano et al., 2002; Gaboriaud et al., 2003, 2004; Bally et al., 2009; Brier et al., 2010), these locations have direct functional implications in terms of C1 activation. Thus, the X-ray structure of the zymogen C1r catalytic domain has led to the conclusion that an outward movement of the C1q stems is necessary to disrupt the resting head-to-tail dimeric structure of C1r and thereby trigger C1 activation (**Figures 5B–D**). If this assumption is correct, any ligand recognized through the outer part of the C1q globular domain, as proposed for IgG (**Figure 5A**), is expected to trigger efficient C1 activation (**Figure 5F**). Conversely, given the inner positioning of their interaction sites, binding of ligands such as phosphoserine, deoxy-D-ribose, and heparan sulfate would be unable to generate the appropriate outward movement of the C1q stems, particularly if these ligands are clustered in dense surface patches, thus preventing or restraining activation of the C1 complex. In full agreement with this hypothesis, it has been shown that immune complexes trigger efficient activation of C1 in the presence of C1 inhibitor, in contrast to DNA and heparin (**Figure 5E**; Ziccardi, 1982; Garlatti et al., 2010).

CONCLUSION

Uncontrolled activation of the classical complement pathway is involved in many inflammatory pathologies. Conversely, C1q deficiency is associated with major defaults in the uptake of apoptotic cells and correlates with autoimmune diseases such as systemic lupus erythematosus and glomerulonephritis, emphasizing the crucial role of C1q in the maintenance of immune tolerance (Botto and Walport, 2002). Whereas heparan sulfate and other sulfated molecules are known to inhibit complement activation, DNA, and phosphatidylserine are both eat-me signals involved in the removal of apoptotic cells by C1q. It is tempting to hypothesize that, because the binding sites for these ligands are all located within the same area of the C1q globular domain, on the inner edge of the C1q cone, their recognition by C1q only generates low C1 activation, hence contributing to the control of the inflammatory reaction, in addition to complement regulators (Zipfel and Skerka,

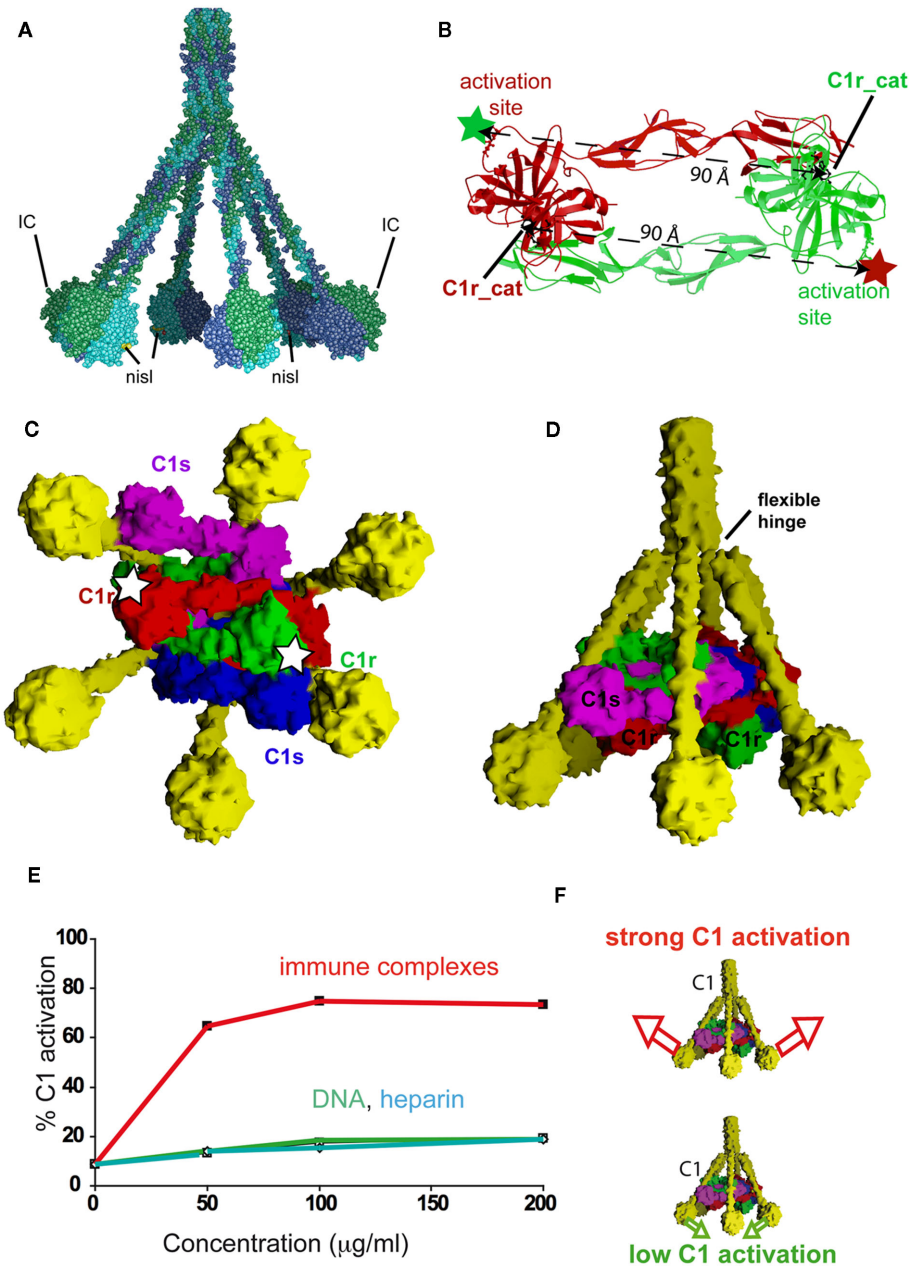

FIGURE 5 | Implications on C1 activation of the location of ligand-binding sites in the C1q globular domain. (A) Location of the ligand-binding sites according to the current version of the C1q model. Nisl: non-immune self ligands binding area as described in **Figure 4**; IC: proposed binding sites for IgG-containing immune complexes. **(B)** Dimeric structure of the C1r catalytic domain in its "resting state." In this configuration, a distance of 90 Å is observed between the catalytic site (C1r_cat) of one subunit and the activation site (colored star) to be cleaved in the other subunit. Such a distance prevents unwanted spontaneous C1r autoactivation, which requires transient disruption of the dimer. **(C,D)** Bottom and side views, respectively, of the current C1 model (Bally et al., 2009; Brier et al., 2010). In **(C)** white stars show the approximate positions of the C1r activation sites. **(E)** C1 activation by IgG-containing immune complexes, heparin and DNA. **(F)** Schematic interpretation of **(E)**. According to our hypothesis, a strong outward movement of the C1q stems (red arrows) induced by binding to immune complexes is expected to disrupt the C1r catalytic dimer and thereby induce C1 activation. In contrast, binding to non-immune self ligands would generate little or no activation (green arrows). (Modified from Budayova-Spano et al., 2002 and Garlatti et al., 2010).

2009). Recently, another molecule from self, heme, was shown to bind C1q and inhibit C1 activation by immune complexes and C-reactive protein (Roumenina et al., 2011). Interestingly, one of the two heme binding sites postulated by these authors is located in subunit C of the C1q globular domain, in the vicinity of the sites identified for DNA, phosphatidylserine, and heparan sulfate, suggesting that heme-mediated inhibition of C1 activation could involve a similar mechanism.

It is clear, however, that this scheme does not apply to all known non-immune C1q self ligands. Thus, whereas the oxidized form of low-density lipoprotein (ox-LDL) does not trigger C1 activation despite its ability to bind C1q with high-affinity (Biro et al., 2007), the enzymatically modified species (E-LDL) is a potent C1 activator (Biro et al., 2007, 2010). In the same way, cardiolipin, β-amyloid fibrils, as well as different forms of the prion protein have been shown to bind C1q and activate C1 to various extents (Peitsch et al., 1988; Tacnet-Delorme et al., 2001; Dumestre-Pérard et al., 2007; Sim et al., 2007; Sjöberg et al., 2008). Further investigations at the molecular level will be necessary to generate a more detailed map of the ligand-binding sites of C1q and uncover all the secrets of this unique sensor molecule. Such advances would have important potential therapeutic applications, considering that ligand recognition by C1q may elicit both beneficial and deleterious effects, as exemplified in neurodegenerative diseases where both types of effects have been reported (van Beek et al., 2003; Veerhuis et al., 2011). Ideally, the aim would be to modulate the classical complement pathway by preventing or limiting its noxious effects while preserving its protective role. In the long term, this goal should be achievable through the design of inhibitory molecules able to specifically target the strong C1-activating binding sites located on the outer part of the C1q globular domain, while preserving the functionality of the inner binding area allowing recognition and efficient clearance of altered self ligands.

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The C1q family of proteins: insights into the emerging non-traditional functions

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Research conducted over the past 20 years have helped us unravel not only the hidden structural and functional subtleties of human C1q, but also has catapulted the molecule from a *mere* recognition unit of the classical pathway to a well-recognized molecular sensor of damage-modified self or non-self antigens. Thus, C1q is involved in a rapidly expanding list of pathological disorders – including autoimmunity, trophoblast migration, preeclampsia, and cancer. The results of two recent reports are provided to underscore the critical role C1q plays in health and disease. *First* is the observation by Singh et al. (2011) showing that pregnant C1q−/− mice recapitulate the key features of human preeclampsia that correlate with increased fetal death. Treatment of the C1q−/− mice with pravastatin restored trophoblast invasiveness, placental blood flow, and angiogenic balance and, thus, prevented the onset of preeclampsia. *Second* is the report by Hong et al. (2009) which showed that C1q can induce apoptosis of prostate cancer cells by activating the tumor suppressor molecule WW-domain containing oxydoreductase (WWOX or WOX1) and destabilizing cell adhesion. Downregulation of C1q on the other hand, enhanced prostate hyperplasia and cancer formation due to failure of WOX1 activation. C1q belongs to a family of structurally and functionally related TNF-α-like family of proteins that may have arisen from a common ancestral gene. Therefore C1q not only shares the diverse functions with the tumor necrosis factor family of proteins, but also explains why C1q has retained some of its ancestral "*cytokine-like*" activities. This review is intended to highlight some of the structural and functional aspects of C1q by underscoring the growing list of its non-traditional functions.

Keywords: role of C1q in inflammation and autoimmunity, C1q and cancer, emerging role of C1q, functions of C1q

INTRODUCTION

The first component of complement, C1, is a multimolecular complex comprising of C1q and a Ca^{2+}-dependent tetramer, $C1r_2$–$C1s_2$. The role of C1q within this complex is to serve as a recognition signal – a signal that is readily translated into a highly specific and orderly intramolecular rearrangement resulting in a sequential proteolytic activation – first of C1r and then of C1s – thereby triggering activation of the classical pathway (Cooper, 1985; Schumaker et al., 1987; Arlaud et al., 2001). However, the role of C1q is not restricted to recognition of immune complexes or other molecules that activate the classical pathway. Evidence accumulated to date clearly demonstrates that C1q is a key player in a rapidly expanding list of pathophysiological conditions which include: trophoblast invasion and placental development (Agostinis et al., 2010), onset of preeclampsia (Singh et al., 2011), regulation of autoimmune diseases such systemic lupus erythematosus (SLE; Walport et al., 1998) and activation of the tumor suppressor molecule WW-domain containing oxydoreductase, which plays a critical role in the pathogenesis of prostate cancer (Hong et al., 2009).

Unlike most of the complement proteins, which by and large are synthesized in the liver, C1q is synthesized extrahepatically by a wide range of cell types including monocytes/macrophages (Bensa et al., 1983; Loos, 1983; Tenner and Volkin, 1986; Kolosov et al., 1996; Armbrust et al., 1997), epithelial cells (Colten, 1976) mesenchymal cells (Morris et al., 1978), dendritic cells (Vegh et al., 2003; Castellano et al., 2004a), trophoblasts (Bulla et al., 2008; Agostinis et al., 2010), microglial cells (Lynch et al., 2004; Farber et al., 2009), fibroblasts (Reid and Solomon, 1977; Skok et al., 1981; Gulati et al., 1993), and endothelial cells (Bulla et al., 2008; Bossi et al., 2011). The synthesized molecule in turn, is either transiently expressed as a cell membrane associated molecular ligand (mC1q; Loos, 1983), or secreted into the pericellular milieu where it can modulate cell-specific biological responses in a manner that involves an autocrine and/or paracrine signaling through cell surface receptors and their signaling partners (Menzies et al., 2008). The diversity of immunological functions mediated by C1q could perhaps be explained by having a unique structural feature: a collagen-like domain (cC1q) and a globular heads domain (gC1q) present in the same protein. This paper will shed some light into the evolution and structural

Abbreviations: cC1q, the collagen domain of C1q; cC1qR, receptor for cC1q; CRT (identical to cC1qR), calreticulin; gC1q, the globular heads of C1q; gC1qR, receptor for gC1q; ghA, ghB, and ghC, globular heads of the A, B, and C chains of C1q respectively.

basis of C1q function and highlight some of its emerging novel functions.

STRUCTURE OF HUMAN C1q

Human C1q (460 kDa) is a collagen-like, structurally complex, hexameric glycoprotein, which associates with the Ca^{2+}-dependent $C1r_2$–$C1s_2$ tetramer (360 kDa) to form the pentameric C1, the first component of the serum complement system (Calcott and Müller-Eberhard, 1972; Reid and Thompson, 1983; Reid, 1989). It is composed of six globular "*heads*" linked via six collagen-like "*stalks*" to a fibril-like central region (Shelton et al., 1972; Svehag et al., 1972; Knobel et al., 1975; Brodsky-Doyle et al., 1976; Sellar et al., 1991). The C1q molecule (**Figure 1**), consists of 3 remarkably similar but distinct polypeptide chains, A, B, and C, each of which occurs 6 times in the molecule to give rise to a total of 18 chains (6A, 6B, 6C; Reid, 1989). The three chains are the product of three distinct genes, which are aligned $5' \Rightarrow 3'$, in the same orientation, in the order A–C–B on a 24-kb stretch of DNA on chromosome 1p (Reid, 1985; Sellar et al., 1991). Each polypeptide chain (A = 28 kDa, B = 25 kDa, C = 24 kDa) consists of 81 amino acid-long collagen-like *N*-terminal "*stalk*" and a *C*-terminal, globular "*head*" of 136 amino acids (Reid, 1989). The chains are arranged to form 6 triple helical strands, three peptide chains, A, B, and C, forming one strand (**Figure 1**). In each strand, the triple helix is formed between the *N*-terminal collagen-like sequences of the three chains while the globular "*head*" is formed from the *C*-terminal portion of these chains (Sellar et al., 1991; Kishore and Reid, 1999). Chains A and B are disulfide-linked within a given strand through formation of disulfide bridges between the half-cysteine residues located at A-4 and B-4, whereas the C chain forms a disulfide bond with the C chain of the next strand through cysteine residues found at position C-4 (Reid, 1989). Thus, the two disulfide-linked strands form a doublet, and three doublets are linked by non-covalent forces to give C1q its signature electron microscopic structure resembling a bouquet of tulips (Shelton et al., 1972; Brodsky-Doyle et al., 1976; **Figure 1**). Approximately 8% (w/w) of human C1q is carbohydrate, which is in the form of glucosylgalactosyl disaccharide units linked to hydroxylysine residues in the cC1q domain, and six asparagine-linked sugar chains located in the gC1q domain (Yonemasu et al., 1971; Calcott and Müller-Eberhard, 1972; Reid et al., 1972). The two types of sugars in turn account for approximately 69 and 31% of the total carbohydrate respectively (Mizuochi et al., 1979; Shinkai and Yonemasu, 1979; Reid, 1989).

From a structure–function point of view, the C1q molecule contains two very well defined major functional domains through which it enters into a wide range of molecular interactions. These domains are known as the *collagen-like domain (cC1q)*, and the *globular "heads" domain (gC1q)*. Of these, the gC1q domain, which recognizes a wide range of molecular patterns including modified-self and non-self antigens, is considered to be key to the versatility of C1q function (Gaboriaud et al., 2009). The two domains of C1q can be purified separately after the intact C1q molecule is first subjected to either peptic digestion at pH 4.45, or collagenase digestion

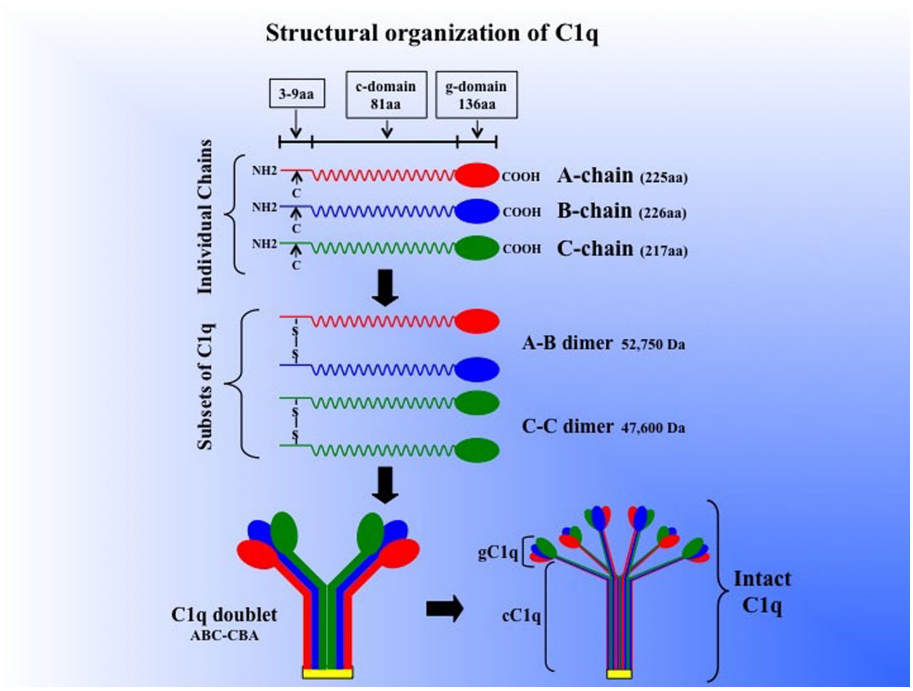

FIGURE 1 | Schematic model of the subunit structure and assembly of intact of C1q. Three similar chains – A, B, and C – are assembled to form disulfide-linked A–B and C–C dimers. One strand of the molecule consists of an A–B dimer non-covalently linked to a C chain forming (A–B–C). The C chain of one strand is then disulfide-linked to the C chain of a neighboring strand to give an A–B C–C B–A doublet and three such doublets are linked by non-covalent forces giving C1q its final signature "bouquet-like" structure. The length of each chain as well as the length of the gC1q and cC1q domains (for brevity, g- or c-domain) is given in parenthesis. (The figure was adapted from Reid, 1989).

at near neutral pH 7.4 (Knobel et al., 1974). The respective purified heterotrimeric domains can then be used in functional studies, although the affinity of the individual trimeric gC1q domain is likely to be several-fold less than that of the intact C1q because of the multivalency of the latter (Tacnet et al., 2008). Evidence accumulated to date convincingly shows that the two domains of C1q participate in a multiplicity of immunological functions by serving as a binding sites for a long list of pathogen-associated molecular patterns (PAMPs) as well as danger associated molecular patterns (DAMPs). These include viral proteins such as gp41 (Ebenbichler et al., 1991), acute phase proteins such as C-reactive protein (Jiang et al., 1991; Gewurz et al., 1993), bacterial and mitochondrial membranes (Gewurz et al., 1993), DNA (Jiang et al., 1991), as well as β-amyloid peptide (Jiang et al., 1994; Tacnet-Delorme et al., 2001). The gC1q domain has a heterotrimeric structure composed of the C-terminal halves of each of the three chains A, B, and C, and each of these heterotrimeric structures are postulated to function independently (Kishore and Reid, 1999, 2000). Because of the availability of recombinant forms of each of the globular heads (gh) or modules, it has now been possible to purify the individual modules (ghA, ghB, and ghC) as recombinant proteins and study their individual roles in ligand binding and biological function (Kishore et al., 2003; Ghai et al., 2007). Indeed, mutational studies using ghA, ghB, and ghC modules have identified the gh residues involved in recognition of IgG and IgM (Kojouharova et al., 2004; Gadjeva et al., 2008). In addition to recognition of self and non-self antigens, C1q can enter into a vast array of interactions by binding to cell surface molecules either via its cC1q or gC1q domain. C1q receptors mediate a plethora of immunologic functions including phagocytosis, and clearance of apoptotic cells (Bobak et al., 1987; Nauta et al., 2002; Païdassi et al., 2011).

Although the list of cell surface or cell associated molecules recognized by C1q is increasing, there are at least two well characterized and ubiquitously distributed cell surface molecules – known respectively as cC1qR and gC1qR – that recognize the two domains of C1q (Ghebrehiwet and Peerschke, 2004a). Although each of these molecules lacks a consensus sequence motif for a traditional transmembrane segment, the lack of direct conduit to intracellular signaling elements is circumvented by the formation of a docking/signaling complex with transmembrane proteins. This partnership in turn is dictated by the specific biological response to be induced (Ghebrehiwet et al., 2001; Ghebrehiwet and Peerschke, 2004a). Each of these receptors is a multiligand binding protein capable of inducing a wide range of cellular functions (Ghebrehiwet and Peerschke, 2004a). More importantly, binding of C1q to cell surfaces via either receptor induces a plethora of biological functions that play a critical role in innate and adaptive immunity, inflammation, infection, and cancer (Ghebrehiwet et al., 2001).

THE C1q FAMILY OF PROTEINS

From data available to date, it has become clear that the unusual feature of having a *collagen-like* and *globular region* in the same protein is not unique to C1q. Instead, the data show that C1q belongs to a long list of oligomeric proteins that are structurally and functionally similar. These proteins, whose members include: *mannan-binding lectin* (*MBL*), *lung surfactant protein A* (*SP-A*), SP-D, *collectin-43* (*CL-43*), collectin-46 (CL-46), and *bovine conglutinin* (Malhotra et al., 1992) are collectively known as collectins (*col*lagen containing *lectins*) by virtue of having collagen-like sequences contiguous with non-collagen-like stretches (Hoppe and Reid, 1994). Collectins are calcium dependent molecules and belong to a group III of the family of lectins containing C-type carbohydrate recognition domains (CRD; Lu et al., 1993; Hakansson and Reid, 2000). Their genes characteristically contain a small exon encoding a 36 amino acid-long neck region, which links the collagen domain with the CRD. The CRD in turn, is responsible for recognition of glycoconjugates containing mannose and fucose on microorganisms but *not* on self-proteins, resulting in enhanced clearance of pathogenic microorganisms (Hakansson and Reid, 2000). Unlike the collectins however, C1q does not possess a consensus domain for CRD, but it shares another common feature with the collectins in that it possesses galactose–glucose disaccharides attached to the collagenous regions of each protein (Mizuochi et al., 1979; Reid, 1979; Shinkai and Yonemasu, 1979). Despite lacking a CRD domain however, recent data suggest that C1q also has lectin-like activity suggesting that carbohydrates – especially those on pathogens and apoptotic cells – may provide additional binding targets where the binding of C1q is critical for removal of self waste (Païdassi et al., 2008).

In addition to the collectins, several other molecules, which are structurally similar to C1q, have also been described. One of these is, adipocyte complement-related protein of 30 kDa (ACRP30) or adiponectin (Scherer et al., 1995; Hu et al., 1996; Berg et al., 2001; Wong et al., 2004). ACRP30 or adiponectin, is secreted exclusively from fat cells and implicated in energy homeostasis and obesity as well as enhancement of hepatic insulin action (Berg et al., 2001; Wong et al., 2004). It is a protein of 247 amino acids consisting of an *N*-terminal collagen-like domain and a *C*-terminal globular domain (Scherer et al., 1995; Berg et al., 2001). The globular domain shares a significant homology to the globular domains of type VIII and X collagens as well as subunits of C1q. More intriguing however is the fact that the crystal structure of a homotrimeric fragment from ACRP30 revealed an unexpected homology to the tumor necrosis factor (TNF) family of proteins. Because of identical folding topologies, key residue conservations, and similarity of trimer interfaces and intron positions, these studies have firmly established an evolutionary link between the TNF-α and the C1q family of proteins suggesting that TNF arose by divergence from an ancestral recognition molecule of the innate immune system (Shapiro and Scherer, 1998). The evolutionary connection between C1q-like proteins and TNF-α, which control many aspects of *inflammation*, adaptive immunity, *apoptosis*, and energy homeostasis, not only sheds insight into the shared diverse functions of this family of proteins (Shapiro and Scherer, 1998), but also explains why C1q has retained some of its "*cytokine-like*" activities. Structurally, the C1q/TNF family of proteins contains the same compact "jellyroll" β fold, usually organized as trimers but with diverse functions ranging from cytokine activity (TNF-α), regulation of metabolism (ACRP30), connective tissue organization (collagen type VIII and X), cellular activation (CD40L), and innate immunity (C1q; Kishore et al., 2002, 2004; Gaboriaud et al., 2007). More recently, the crystal structure of the gC1q domain of C1q was solved and the structure reveals a compact, almost spherical heterotrimeric assembly held together mainly by non-polar

interactions, with a Ca^{2+} ion bond at the top of the molecule (Gaboriaud et al., 2009). More importantly, the structural studies revealed that the heterotrimeric assembly of the gC1q domain appears to have unique features that allow its versatility (Gaboriaud et al., 2009). Other molecules in which the "gC1q domains" are found at the C-terminal end include: precerebellin (Kavety et al., 1994), multimerin (Hayward et al., 1995), and types VIII and X collagens (Bogin et al., 2002). Therefore, despite its similarities with the collectins, the structural folds of the gC1q domain, which are more similar to the TNF than to the CRD region of the collectins – make C1q more related to the TNF family of proteins (Kishore et al., 2004).

The significance of a particular human protein is sometimes underscored by the fact that pathogenic microorganisms specifically mimic its functional domain or antigenic determinant for the purpose of immune evasion and thus enhance their survival. For example, the three-dimensional structure of the major surface protein of *Bacillus anthracis* spore surface protein, bacillus collagen-like protein of anthracis (BclA) reveals remarkable similarity to C1q – although there is only low sequence identity between the two proteins (Sylvestre et al., 2002; Rety et al., 2005). Furthermore, both BclA and C1q have been shown to bind to lung alveolar surfactant component-SP-C, suggesting that both recognize common targets in the alveolar component (Rety et al., 2005). This molecular mimicry in turn, underscores not only the functional relevance of the gC1q domain, but also pathogenic microorganisms mimic this signature domain in order to get access into host cells. The exosporium of *B. cereus*, which belongs to the same genus of spore-forming aerobic bacteria as *B. anthracis*, contains a binding site for gC1qR. Although the nature of the *B. cereus* molecule has not been as yet identified, the fact that it recognizes gC1qR on cells for the initial spore attachment and entry suggests that this molecule may also contain a gC1q-like domain (Ghebrehiwet et al., 2009).

THE DIVERSE BIOLOGICAL FUNCTIONS OF C1q

Although for many years, the traditionally accepted role of C1q is the recognition of immune complexes and activation of the classical pathway, there is an ever-growing body of evidence, which suggests that C1q is a major sensor of DAMPs and PAMPS and plays a critical role in inflammation as well as innate and adaptive immunity (**Figure 2**) (Kojouharova et al., 2003; Sontheimer et al., 2005; Ghai et al., 2007; Gadjeva et al., 2008; Païdassi et al., 2008; Hosszu et al., 2010). In addition, C1q induces a plethora of immunologic functions by interaction with distinct cell surface molecules, which recognize either, its gC1q or cC1q domain (Ghebrehiwet and Peerschke, 2004a). These cell surface molecules include cC1qR or calreticulin (CR), gC1qR (Malhotra et al., 1992; Ghebrehiwet and Peerschke, 2004a) CD93 (Nepomuceno et al., 1997; Steinberger et al., 2002), as well as other associated adapter molecules such as CD91 (Ogden et al., 2001; Gardai et al., 2003), CD44, DC-SIGN, and β1 integrin (Feng et al., 2002; Menzies et al., 2008). Interaction of C1q with cell surface molecules, induces a wide range of biological responses including stimulation of leukocyte oxidative response (Tenner and Cooper, 1982), phagocytosis (Bobak et al., 1987), suppression of B and T cell proliferation (Ghebrehiwet et al., 1990), fibroblast and endothelial cell adhesion (Bordin et al., 1990; Chen et al., 1994; Feng et al., 2002), trophoblast cell migration (Agostinis et al., 2010), regulation of dendritic cells (Castellano et al., 2004a,b; Hosszu et al., 2010), angiogenesis (Bossi et al., 2011), and chemotaxis of eosinophils, mast cells, neutrophils, and dendritic cells (Ghebrehiwet et al., 1995; Kuna et al., 1996; Leigh et al., 1998; Vegh et al., 2005). Another interesting aspect of the C1qRs, is that both are released into the pericellular milieu, by proliferating, activated, or proinflammatory cells. Because both are capable of activating the classical pathway, it is believed that the secreted molecules may exacerbate or trigger the local inflammatory process. The binding site for gC1qR on C1q has been shown to be on the A-chain of C1q (Ghebrehiwet et al., 2001), which is the same or overlapping site to a putative IgG site (Marques et al., 1993).

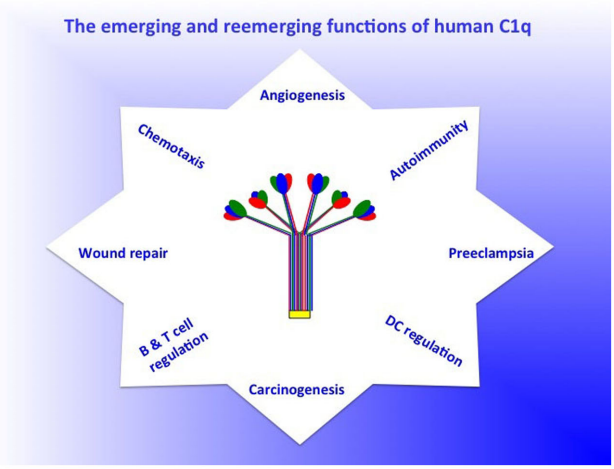

FIGURE 2 | The emerging and reemerging functions of C1q. The figure is intended not to a representative of the plethora of C1q-mediated functions, but rather is intended to highlight the growing list of emerging and reemerging non-traditional functions of C1q. For a more or less complete list of C1q functions see recent paper by Nayak et al. (2010).

C1q DEFICIENCY AND AUTOIMMUNITY

The significance of C1q in health and disease is further underscored by the fact that deficiency in C1q leads to a plethora of diseases including recurrent infections, otitis media, meningitis, pneumonia (Nishino et al., 1981; Komatsu et al., 1982) as well as autoimmune diseases, a prototype of which is SLE. In fact, deficiency of C1q is considered to be a strong susceptibility factor for SLE as evidenced by the fact that almost all (≥92%) of the known patients with C1q deficiency have developed the disease (Botto et al., 1998; Walport et al., 1998; Pickering et al., 2000). The significance of C1q in SLE is further substantiated by the finding that, peripheral blood monocytes from SLE donors, who are not C1q deficient, have a significantly impaired ability to upregulate C1q synthesis both at the mRNA and protein levels, when compared to their healthy counterparts (Moosig et al., 2006). Furthermore, previous studies have shown SLE patients to synthesize high levels of a non-functional, low molecular weight form of C1q, that run with an apparent molecular weight of 150 kDa by gel filtration (Hoekzema et al., 1985). This non-functional form of C1q

is characterized by having an abnormal A–C disulfide bonding instead of the normal pattern, which is A–B and C–C (Hoekzema et al., 1985, 1987, 1989). Although this form of C1q retains affinity for the $C1r_2C1s_2$-complex, it does not bind to the Fc in IgG aggregates (Martin and Loos, 1988). Because cultured monocytes from normal individuals can synthesize both the dysfunctional as well as the normal forms of C1q, it is hypothesized that in disease states such as SLE, a condition may exist which favors a disproportionate concentration of dysfunctional C1q, especially since the normal C1q would be depleted due to complement activation (Hoekzema et al., 1985). Although the molecular underpinnings are still being worked out, two hypotheses have been put forth to explain the role of C1q in SLE. The *first* deals with the role of C1q in removal of apoptotic waste. Removal of apoptotic cells and cellular debris is essential for maintenance of tissue homeostasis, organogenesis, remodeling, development, and maintenance of the immune system, protection against neoplasia, and resolution of inflammation. The mechanism of this removal involves the recognition of the apoptotic cell surface and initiation of phagocytic uptake by macrophages and dendritic cells. C1q, has been shown to play a major role both in the recognition and removal of apoptotic cells (Casciola-Rosen et al., 1994; Korb and Ahearn, 1997) 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 (phosphatidylserine, DNA, and possibly gC1qR; Païdassi et al., 2008). This in turn implies that defective clearance of immune complexes and apoptotic cells, leads to immune recognition of hidden epitopes – a critical immunopathogenic event leading to the development of SLE, the prototype autoimmune disease. Indeed, C1q directly and specifically binds to antigens expressed early on the surface of apoptotic cells through its gC1q domain (Korb and Ahearn, 1997; Païdassi et al., 2008) and the common autoantigens targeted in SLE, such as phosphatidylserine and DNA are found in high concentrations on the surface of apoptotic cells (Casciola-Rosen et al., 1994). However, while its role in removal of apoptotic debris is clearly important, there are a number of redundant pathways involved in the removal of self waste, thus making the role of C1q not as critical (Ghebrehiwet and Peerschke, 2004b). The *second* hypothesis stipulates that autoimmunity arising from the absence or inefficient C1q function, especially at local sites, reflects an important role in maintaining tolerance (Ghebrehiwet and Peerschke, 2004b; Hosszu et al., 2008, 2010). Thus, while C1q could provide active protection from autoimmunity by silencing or regulating autoreactive immune cells, its absence or defective secretion could lead to a loss of peripheral tolerance as a sum total of impaired apoptotic cell clearance and negative signaling (Hosszu et al., 2010). Inherent in this postulate is the notion that the unique structure of C1q, which allows it to interact with its receptors via either its cC1q or gC1q domain, may control the transition from the monocyte state (innate immunity) toward the professional antigen presenting cell (APCs) state (adaptive immunity). Therefore the recent observation showing that C1q functions as a "*molecular switch*" during the narrow window of monocyte to DC transition (Hosszu et al., 2008, 2010), not only explains why C1q is primarily synthesized by potent APCs, but also underscores why its absence can impair antigen uptake and tolerance.

LOCAL SYNTHESIS AND FUNCTION OF C1q

As described above, most inflammatory cells such as those found in atherosclerotic plaques (Peerschke et al., 2004) or cells that undergo phenotypic differentiation or proliferation are known to express and/or secrete C1q (Vegh et al., 2003; Bulla et al., 2008; Hosszu et al., 2008). The emerging postulate is therefore, that locally secreted C1q modulates cellular function (s) by an autocrine and/or paracrine signaling mechanism. For example, fetal trophoblast cells invading the decidua in the early stages of pregnancy not only actively synthesize and express C1q on their surface, but this C1q is actively involved in promoting trophoblast invasion of the decidua (Agostinis et al., 2010). This in turn is mediated by two cell surface receptors – gC1qR and $\alpha_4\beta_1$ integrin, which promote trophoblast adhesion and migration through the activation of ERK1/2 MAPKs (Agostinis et al., 2010). In support of this is the finding that C1q−/− mice manifested increased frequency of fetal resorption, reduced fetal weight, and smaller litter size when compared to their wild-type counterparts suggesting that defective local production of C1q may be involved in pregnancy disorders such as preeclampsia (Agostinis et al., 2010). The significance of C1q in pregnancy disorders is further supported by an independent study, which showed that pregnant C1q−/− mice express the key features of human preeclampsia that correlate with increased fetal death (Singh et al., 2011). Treatment of C1q−/− mice with pravastatin restored trophoblast invasiveness, placental blood flow, and angiogenic balance and, thus, prevented the onset of preeclampsia (Singh et al., 2011). It is also important to note within this context, the results of recent studies, which showed that *in vivo*, C1q induces angiogenesis and promotes wound healing. This in turn identifies not only a novel role but also identifies C1q as a potential pro-angiogenic molecule for the treatment of wounds and chronic ulcers (Bossi et al., 2011).

THE EMERGING ROLE OF C1q IN CARCINOGENESIS

Another emerging role of C1q is in the regulation of cancer cell survival and progression. Recent studies have shown that C1q, which is expressed on basal and epithelial cells of prostate tissues, sustains the activation of the tumor suppressor WOX1 (WW-domain containing oxydoreductase), which is needed for blocking cancer cell proliferation. This suggests that C1q may induce apoptosis of prostate cancer cells by activating WOX1 and destabilizing cell adhesion. Conversely, downregulation of C1q was found to enhance prostate hyperplasia and cancerous formation due to failure of WOX1 activation (Hong et al., 2009). More importantly, although C1q is expressed in the normal prostate, it is significantly reduced in benign prostatic hyperplasia and prostate cancer tissues (Hong et al., 2009). Therefore, the C1q/WOX1-induced death of cancer cells could have a universal implication in cancer, as long as C1q and WOX1 are present in both the extracellular and intracellular milieu, and are sufficient in activating the apoptotic cascade (Hong et al., 2009). However, since the autocrine or paracrine signaling effect of locally expressed C1q is most likely going to be in concert with its cell surface receptors and signaling partners, it is important to note that both cC1qR/calreticulin (cC1qR/CRT) and gC1qR have also been implicated in carcinogenesis, albeit their roles seem to be rather divergent. Whereas cC1qR/CRT is reported to have tumor suppressive effect, gC1qR appears to be

requisite for tumor cell progression and metastasis. For example, when Epstein–Barr virus (EBV)-immortalized B lymphocytes were injected into nude mice, they were found to reproducibly inhibit tumor growth (Tosato et al., 2003). This anti-tumor activity, which is released by the lymphoblastic cells, was later identified to be a fragment of cC1qR/CRT corresponding to the N-terminal residues 1–180. Subsequently, recombinant protein corresponding to this N-terminal fragment was found to inhibit endothelial cell proliferation *in vitro* and angiogenesis *in vivo* (Tosato et al., 2003). The inhibition of endothelial cell proliferation was due to direct binding of the cC1qR/CRT fragment to the extracellular matrix protein, laminin thereby interfering with endothelial cell attachment. Further studies showed that subcutaneous inoculation of recombinant CRT fragment into nude mice not only prevented the development of subcutaneous tumors, but also inhibited the growth of established ones (Tosato et al., 2003). Since the collagen tail of C1q (cC1q) binds to the N-terminal half of cC1qR/CRT, which contains several short (7–10 amino acids) CH2-like motifs (ExKxKx) similar to the C1q binding motif found in the CH2 domain of IgG (Sontheimer et al., 2003), these C1q binding regions of cC1qR/CRT have the potential to be developed into therapeutic modalities.

The receptor for the ghs of C1q, gC1qR, on the other hand, may have functions that enhance tumor growth and tumor progression. First, histochemical staining with anti gC1qR mAb revealed marked differential expression of gC1qR in thyroid, colon, pancreatic, gastric, esophageal, and lung adenocarcinomas compared to the non-malignant histologic counterparts. In contrast, differential expression was not observed in endometrial, renal, and prostate carcinomas (Rubinstein et al., 2004; Peerschke et al., 2011). Recent studies not only corroborated these findings, but additionally showed that cell surface expressed gC1qR is a receptor for the tumor homing peptide, LyP-1 (Fogal et al., 2008). Moreover, recent studies using human lung carcinoma A549 cells have shown the cell surface gC1qR to be a key regulator for lamellipodia formation and cancer metastasis via receptor tyrosine kinase activation. Furthermore, the growth factor-induced lamellipodia formation and cell migration were significantly decreased in gC1qR-depleted cells, with a concomitant blunt activation of the focal adhesion kinase and the respective receptor tyrosine kinases (Kim et al., 2001). These studies collectively suggest that gC1qR, which is highly upregulated on the surface of tumor cells and inflammatory cell scan serve not only as a tumor cell marker, but also can be a target for therapeutic intervention (Peerschke et al., 2011).

CONCLUDING REMARKS

Because of the incredible number of reports that have come out in the past 20 years, it is not possible to list all the functions in which C1q is involved. Therefore, while touching on some of the well known functions of C1q, this paper underscores the role of C1q in novel and emerging areas of diseases (e.g., preeclampsia, cancer, and wound healing) in which either the soluble form or the membrane associated form of C1q appear to be intimately involved. The chimeric structure of C1q – containing two functional domains – together with the fact that it is synthesized by a wide range of proinflammatory cell types makes it likely that it is a potent orchestrator of molecular pathways involved not only in innate and adaptive immunity, but also in a wide range of inflammatory diseases including cancer. The heterotrimeric gC1q domain of C1q is also revealing itself as a powerful pattern recognition domain with the ability to sense a broad range of ligands – including a number of altered self-motifs and pathogen-associated molecular ligands – each of which will likely trigger a multiplicity of immunologic functions, which are either beneficial or deleterious to the host. More than ever, the availability of recombinant forms of the heterotrimeric trimeric gC1q domains will undoubtedly help us gain insight into the mechanism of interaction between C1q and the vast array of molecular patterns. The best is yet to come.

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The EMILIN/multimerin family

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Elastin microfibrillar interface proteins (EMILINs) and Multimerins (EMILIN1, EMILIN2, Multimerin1, and Multimerin2) constitute a four member family that in addition to the shared C-terminus gC1q domain typical of the gC1q/TNF superfamily members contain a N-terminus unique cysteine-rich EMI domain. These glycoproteins are homotrimeric and assemble into high molecular weight multimers. They are predominantly expressed in the extracellular matrix and contribute to several cellular functions in part associated with the gC1q domain and in part not yet assigned nor linked to other specific regions of the sequence. Among the latter is the control of arterial blood pressure, the inhibition of *Bacillus anthracis* cell cytotoxicity, the promotion of cell death, the proangiogenic function, and a role in platelet hemostasis. The focus of this review is to highlight the multiplicity of functions and domains of the EMILIN/Multimerin family with a particular emphasis on the regulatory role played by the ligand–receptor interactions of the gC1q domain. EMILIN1 is the most extensively studied member both from the structural and functional point of view. The structure of the gC1q of EMILIN1 solved by NMR highlights unique characteristics compared to other gC1q domains: it shows a marked decrease of the contact surface of the trimeric assembly and while conserving the jelly-roll topology with two β-sheets of antiparallel strands it presents a nine-stranded β-sandwich fold instead of the usual 10-stranded fold. This is likely due to the insertion of nine residues that disrupt the ordered strand organization and forma a highly dynamic protruding loop. In this loop the residue E933 is the site of interaction between gC1q and the α4β1 and α9β1 integrins, and contrary to integrin occupancy that usually upregulates cell growth, when gC1q is ligated by the integrin the cells reduce their proliferative activity.

Keywords: α4β1 integrin, gC1q-dependent cell adhesion, cell migration, gC1q NMR solution structure, EMI domain, extracellular matrix, skin homeostasis

INTRODUCTION

The elastin microfibrillar interface protein (EMILIN) and Multimerin family of glycoproteins is part of the large superfamily of collagenous and non-collagenous proteins containing the gC1q signature (Shapiro and Scherer, 1998; Doliana et al., 1999; Kishore et al., 2004). A phylogenetic analysis of gC1q sequences of the 32 proteins present in the human genome carrying this domain (Tom Tang et al., 2005; Ghai et al., 2007), show that EMILINs/Multimerins form a small family well distinct from the rest of the superfamily. Comparison of the coding sequences of EMILIN/Multimerin family components against *Drosophila melanogaster* and other invertebrate genome database gives no matches, indicating that this gene family emerged during vertebrate evolution, first appearing in lower cordate, where orthologs of EMILIN1, EMILIN2, and MMRN2 have been identified (Mei and Gui, 2008; Milanetto et al., 2008). MMRN1 is present starting from birds, indicating that this member of the family originated much later during vertebrate evolution. The members of the EMILIN/Multimerin family exert a diverse range of functions directly linked to their gC1q domains although several important functions depend on interactions of soluble proteins and/or cellular receptors with other domains. Structure, function, and physiological and pathological consequences of the activity and/or lack of activity of family members will be summarized.

FUNDAMENTAL NOTIONS ABOUT ECM AND INTEGRIN RECEPTORS

The cells of the microenvironment are embedded in a supporting network of extracellular matrix (ECM) constituents that include collagens, elastin, proteoglycans, and glycoproteins (Li et al., 2007; Zhang and Huang, 2011). The ECM does not constitute a mere structural scaffold but it elicits profound influences on cell behavior and affects cell growth, differentiation, motility, and viability (Bissell et al., 2005; Marastoni et al., 2008; Hynes, 2009; Cukierman and Bassi, 2010). The complexity of the microenvironment is further magnified by the property of ECM molecules to function as reservoirs of growth factors, cytokines, matrix metalloproteinases (MMPs), and processing enzymes (Sternlicht and Werb, 2001). The relative availability of these elements may be affected when the ECM rearranges during wound healing or tumor progression.

The cells sense their microenvironment through interactions with specific receptors, the integrins. Integrins, a large family of αβ heterodimeric cell membrane receptors, are key sensory molecules that translate chemical and physical cues from the dynamic constituents of ECM into biochemical signals that regulate many interrelated cellular processes (Legate et al., 2009). Integrins function as mechanotransducers transforming mechanical forces created by the ECM or the cytoskeleton into cell movement (Chen et al., 2004; Hynes, 2009; Schwartz, 2010). Eighteen α and eight β subunits have been so far discovered in mammals and at least 24 different combinations of α/β subunits have been characterized. Integrins display overlapping ligand specificity and many cells express multiple integrin receptors for the same ligand. Ligation of different integrin receptors by a certain ligand may induce different cellular effects in response to ECM ligation. Following integrin ligation a variety of downstream signaling events ensue: for instance, integrins activate survival pathways via the PI3K and MAPK pathways and act as essential cofactors for growth factors stimulation. Elevated integrin expression in the absence of appropriate ligands, or in the presence of natural or synthetic antagonists, promotes apoptosis under otherwise permissive growth conditions. Moreover, integrins coordinate survival or death responses or cell behavior as a function not only of ECM composition but also of its relative stiffness (Levental et al., 2009; Provenzano and Keely, 2011). This dual function provides an elegant mechanism through which tissue-remodeling events may regulate cell death or survival in a temporal, ECM-governed manner (Dupont et al., 2011). Thus, growth factor shortage and deficiency of pro-survival stimuli or absence of integrin activation leads to intracellular signals, mainly Akt down-regulation, which in turn activate a peculiar type of apoptosis in substrate-adherent cells, anoikis (Gilmore, 2005). Similar balances are at work if one considers the other cellular processes regulated by the many integrin–ECM relationships.

THE EMILIN/MULTIMERIN FAMILY OF PROTEINS

Proteins containing the EMI domain, a cysteine-rich sequence of approximately 80 amino acids, define the EMI domain endowed (EDEN) superfamily in mammals (Braghetta et al., 2004). This superfamily comprises seven genes, which can be subdivided into three families on the basis of the major protein domains. The first family is formed by Emu1 and Emu2 genes (Leimeister et al., 2002) that except for the presence of an EMI domain do not share structural similarities with the other EDEN members. The second family comprises only one gene EMILIN-truncated (EMILIN-T) with a structure similar to the third larger family, but lacking the C-terminal globular domain of C1q (gC1q). The third is the EMILIN/Multimerin family. It is characterized by the N-terminal EMI domain, a central part of the molecule formed by a long (approximately 700 amino acids) region with high probability for coiled-coil structures, and a region homologous to the gC1q domain (**Figure 1**). The family includes EMILIN1 (Colombatti et al., 1985; Bressan et al., 1993; Doliana et al., 1999), EMILIN2 (Doliana et al., 2001), Multimerin1 (MMRN1; Hayward et al., 1991, 1995), and Multimerin2 (MMRN2; Sanz-Moncasi et al., 1994; Christian et al., 2001). Apart from MMRN1 that is deposited in the ECM but for the most part is sequestered in megakaryocytes, endothelial cells (ECs), and platelets granules (Adam et al., 2005), the members of the EMILIN family are constituents of the ECM Under normal conditions the expression of each individual gene overlaps with those of some other members of the family; the only exception is apparently the central nervous system, where only EMILIN2 is detected outside blood vessels, at least at the mRNA level (Braghetta et al., 2004). Moreover, EMILIN2 is specifically and abundantly expressed in mouse cochlear basilar membrane (Amma et al., 2003).

DOMAIN ORGANIZATION

The EMI domain (Doliana et al., 2000a) is located at the N-terminus in all the proteins in which it is expressed and displays a high sequence homology ranging from 60 to 70% between the family members. Cysteine-rich repeat modules are present in various ECM proteins, including several constituents or proteins associated with the elastic fiber (Pereira et al., 1993; Sinha et al., 1998; Doliana et al., 2000a). The EMI domain is rather unique: in fact, while most of the cysteine-rich domains described to date contain either six or eight cysteine residues, it has seven cysteine residues located at regular positions, with the exception of MMRN1 in which the second cysteine residue is missing. The specific spacing of cysteines and a number of strongly conserved aromatic and hydrophobic residues represent a diagnostic feature of this sequence. The consensus WRCCPG(Y/F)xGxxC toward the C-terminus of the domain is highly preserved and apparently unique of the EMI domain. Together with the invariable N-terminal position in all proteins discovered to date it clearly distinguishes the EMI domain from other cysteine-rich domain as, for example, the EGF-like that are often embedded within the protein sequence. In the last decade on the basis of reiterated sequence homology searches other proteins including *Drosophila* CED-1, fasciclin, and periostin have been proposed to harbor an EMI domain (Callebaut et al., 2003). However, after careful comparison refined by eye, it appears that these cysteine-rich domains very scarcely conform to the EMI domain consensus motif and should be classified as novel entities.

A few ECM proteins present long domains in which the 3–4–3–4 spacing of hydrophobic residues (heptad repeats) predicts that they form α-helical coiled-coils which result in a rigid rod (Berger et al., 1995; Wolf et al., 1997). The large central regions of all the EMILIN/Multimerin family members shows a very low or undetectable homology at the primary sequence level; however, when analyzed by specific algorithms it reveals a common structural homology as all the members contain several regions in which the probability for coiled-coil formation is very high.

Between the long central coiled-coil part of the molecules and the gC1q C-terminal domain each component of the family presents different sequences that could confer specific structural and/or functional properties. EMILIN1 presents a 90 residues long sequence, including two sequences corresponding to structures referred to as "leucine zipper." Although its precise function has not been determined yet it might contribute to the coiled-coil association in the C- to N-terminal direction for the assembly of a proper EMILIN1 trimer (see below). Right downstream the leucine zipper there is a short collagenic region formed by 17 GXY triplets organized in a trypsin-resistant triple helix (Mongiat et al., 2000). In place of the leucine zipper motif EMILIN2

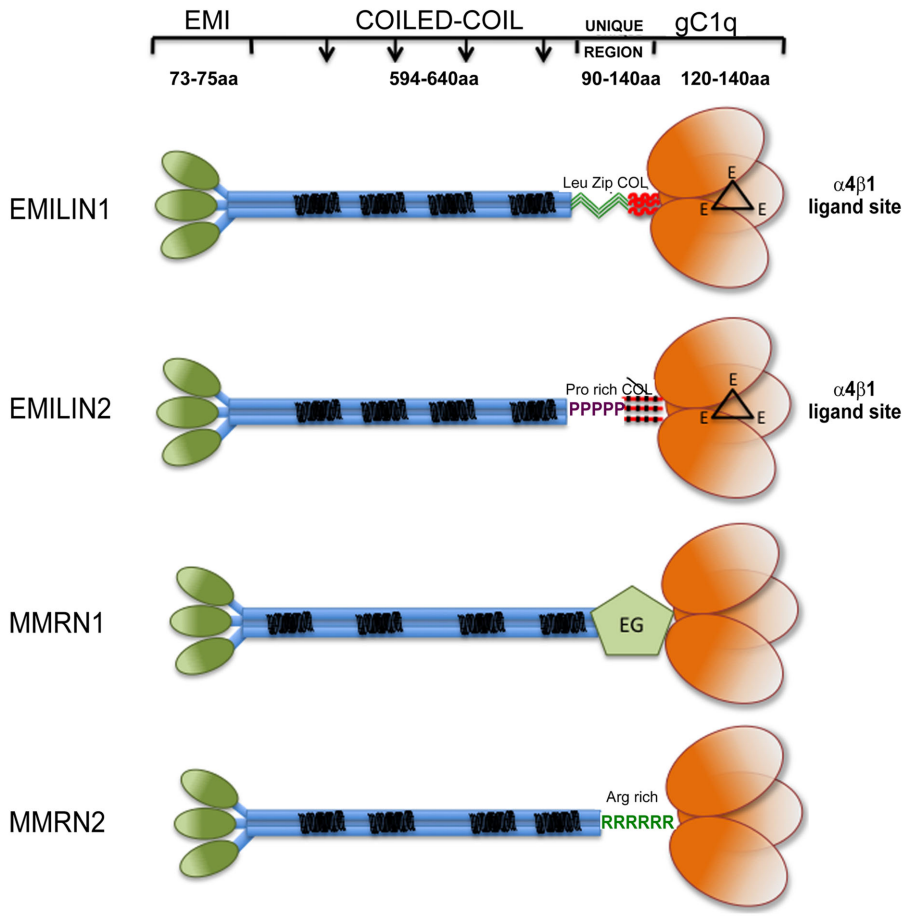
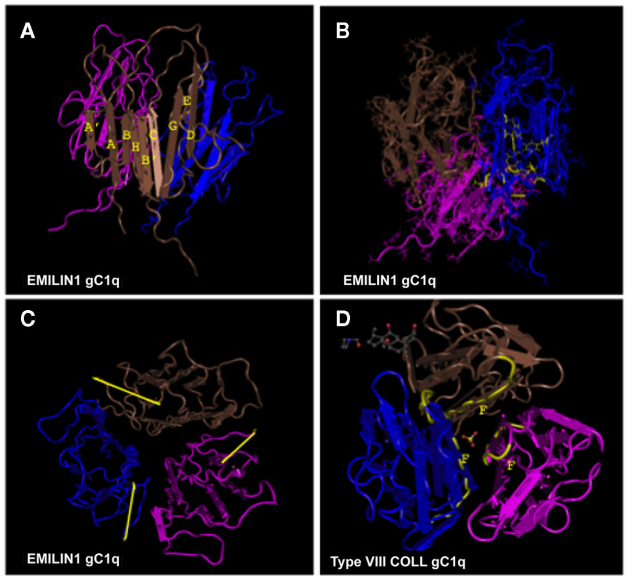


FIGURE 1 | Graphical view of EMILIN/Multimerin family members. Note that: (i) EMI domain: there is some experimental evidence suggesting that EMI domains might self-interact; however, since it is not clear if they form trimers they are depicted separated; (ii) Coiled-coil regions: the similarity between the central regions of the family members is only structural, the heptad repeats are placed in different positions along the primary sequence of the different members of the family. (iii) the so called "UNIQUE REGION" is represented in EMILIN1 by two leucine zippers followed by a functional 17 triplets long collagenic sequence; in EMILIN2 by a proline rich sequence followed by a non-functional 17 triplets long collagenic sequence; in MMRN1 by an arginine rich sequence; in MMRN2 by an EGF-like domain; (iv); the gC1q of EMILIN1 and EMILIN2 possess three unstructured loops (black triangles) each bearing a glutamic acid (E) able to interact with the α4β1 integrin.

harbors a unique proline-rich motif of 53 residues that is highly hydrophilic and exposed to the solvent and thus available for potential interactions with other ligands or provide some flexibility that is not present in EMILIN1. Just upstream of the gC1q domain EMILIN2 also presents a collagenous sequence consisting of 17 triplets like EMILIN1, but with four interruptions, which makes it very unlikely that this sequence could form a stable triple helix. MMRN1 and MMRN2 do not harbor any collagenous sequence. On the contrary, in the corresponding position MMRN1 presents an EGF-like domain and MMRN2 an arginine rich sequence, whose functions are still undisclosed.

The gC1q domain lengths vary between 131 (C1q-C chain of C1q) and 151 (EMILIN1) residues with a high level of conservation of several hydrophobic and uncharged residues. Long before the first crystal structure was resolved Fourier-transform infrared spectroscopy and structure prediction of 15 gC1q sequences suggested a β-sheet secondary structure arrangement for this domain (Smith et al., 1994). The structure was then formally demonstrated by the analysis of the ACRP30/AdipoQ C1q crystal (Shapiro and Scherer, 1998) followed by the solution of the crystal structures of the gC1q of human collagen X (Bogin et al., 2002), mouse collagen VIII (Kvansakul et al., 2003), and human complement gC1q (Gaboriaud et al., 2003). All these crystals show similar quaternary structures obtained by the non-covalent association of three polypeptides whose tertiary structure adopts invariability a beta sandwich topology formed by 10 beta strands organized in two beta sheets. We were then the first to determine the three dimensional NMR solution structure of the human EMILIN1 gC1q homotrimer (Verdone et al., 2004, 2008, 2009). This domain exhibits a striking homology to the gC1q domains of several other members of the C1q/TNF superfamily but, at the same time, displays very peculiar characteristics (**Figure 2**). The quaternary structure of the complex is formed by three mostly identical subunits, apart from very minor differences inherently associated



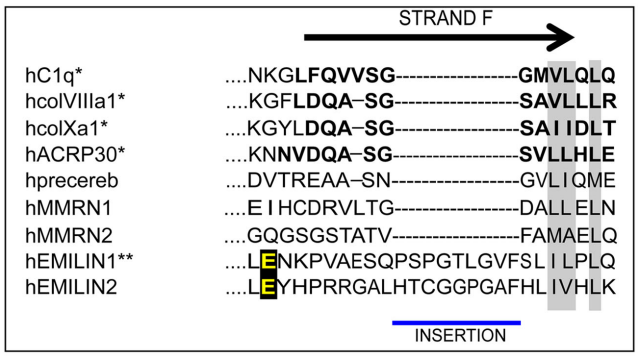


FIGURE 2 | Top: NMR solution structure of the homotrimeric EMILIN1 gC1q domain. The structure was downloaded from database of protein structures maintained at NCBI site (http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid = 68072). Ribbon representation of the assembly, as side view, is presented in **(A)** the three protomers in the trimer are shown in different colors (*pink, blue,* and *brown*). Each monomer has a nine-stranded folding topology, with strands labeled according to the gC1q/tumor necrosis factor superfamily nomenclature. **(B)** The residues highly conserved in all the members of the gC1q superfamily and essential for a correct domain folding are shown in *yellow* only in one monomer for clarity. **(C)** Top view of EMILIN1 gC1q domain. The *yellow* bar highlights the solvent exposed position of the unstructured segment Tyr927–Gly945 **(D)** the X-ray structure of type VIII collagen gC1q, http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid = 25284, showing the buried position of strand F. The same position of strand F is present in the X-ray solution structures of ACRP, type X collagen and Complement gC1q domains. Bottom: Sequence alignment between strand F (residues in bold) of representative members of the C1q superfamily and EMILINs gC1q. *Asterisks* indicate proteins for which the structure has been solved. Similar residues that are conserved in all proteins are shaded in *gray*, and the glutamic acid residue interacting whit α4β1 is in *yellow*.

with the molecular dynamic simulations (Verdone et al., 2008, 2009). Each protomer conserves the typical jelly-roll topology of two β-sheets of antiparallel strands. However, in contrast to all the gC1q structures solved to date, it presents a nine-stranded β-sandwich fold instead of the 10-stranded fold. By sequence homology alignment of C1q family members, the lack of the F beta strand in EMILIN1 gC1q may be due to the insertion of nine residues in the middle of strand F that disrupt its secondary structure. As an important consequence, the highly ordered and rigid strand is substituted by a 19-residues long segment spanning from Y927 to G945. This sequence is unfolded, highly dynamic and highly accessible to solvent, with 10–11 residues protruding from the main globular structure making this region a good candidate for hosting an interaction site. Indeed, by site-directed mutagenesis experiments focused on this segment, we mapped the acidic residue E933 as the site of interaction between gC1q and the α4β1 integrin. This loop is not present in any of the already determined gC1q structures, and seems the only region of gC1q where single mutation or even deletions do not alter the overall domain structure. Interestingly, the quaternary structure of EMILIN1 gC1q assembly locates the unstructured loop at the apex of the homotrimer (**Figure 2D**). Another important information derived from the NMR solution structure of the EMILIN1 gC1q is a marked decrease of the monomer contact surface in the trimeric assembly (3,000 Å^2), compared to ACRP (5,324 Å^2), complement (5,490 Å^2), Type VIII (6,150 Å^2), and type X collagen (7,360 Å^2) gC1q domains. Since the conserved residues are nearly identical in the different gC1q domains, it seems reasonable that the lower thermal stability detected in some of the domains, namely ACRP30 and EMILIN1 depends on the partly different neighboring residues among the various domains. Moreover, the decrease of the buried surface may in part be due to the missing contribution of unstructured element, or even to a contact disturbing activity, as suggested by the decrease of trimer thermal stability observed in some mutants (Doliana et al., unpublished data). Despite various attempts, we were unable to express a soluble form of EMILIN2 gC1q domain and hence to determine its structure, but, based on the sequence homology and functional data, it is conceivable that also the gC1q of EMILIN2 presents the same unstructured loop able to interact with the integrin receptor (see below). None of the other gC1q of the superfamily possess the described unstructured insertion and there are no data on a possible interaction with the α4β1 integrin. It is of note that the E993 residue is the only site of interaction with an integrin localized to date in a gC1q domain.

GENOMIC ORGANIZATION

The EMI domain of EMILIN1 is contained in exons 2 and 3 (Doliana et al., 2000b) and the organization of this domain is remarkably similar to that of EMILIN2 (Doliana et al., 2001). Exon 4 (almost 2000 bp in length) encodes for four coiled-coil structures and two leucine zippers which may represent a sort of functional unit. The short collagenous region of EMILIN1 is encoded by two very short exons (5, 6) and part of exon 7. Finally, the gC1q domain is encoded by part of exon 7 and exon 8. Similarly to EMILIN1 also the EMILIN2 gene consists of eight exons and seven introns. However, whereas the exon structure is remarkably similar between the two genes, several introns of EMILIN2 are much larger than those of EMILIN1 resulting in an overall gene size of about 40 kb as opposed to the remarkably compact EMILIN1 gene (8 kb). Nevertheless, the exon size pattern and location of introns in the coding

sequence are very well conserved between the two genes likely indicating that they evolved from a common ancestor. Whereas most of gC1q domains are encoded within one exon, with the notable exception of cerebellin family where the coding sequence is splitted in three exons, those of EMILIN1 and EMILIN2 are encoded by two exons. MMRN1 and MMRN2 genes have a similar organization with EMILINs genes only limited to the EMI domain and the large exon coding for the coiled-coil structures. At variance, MMRNs present two additional short exons interposed between the EMI domain coding exons and the coiled-coil coding exon. In both genes the gC1q domain is encoded within a single exon.

Before describing in more detail the functions of EMILIN1/Multimerin family formally associated with the gC1q domain, a short summary of the various functions of EMILIN/Multimerin proteins not directly dependent gC1q will follow (**Figure 3**).

gC1q-INDEPENDENT FUNCTIONS

EMILIN1 AND BLOOD PRESSURE CONTROL

Arterial blood pressure is a function of the vasculature resistance and of the cardiac output, i.e., the amount of blood pumped out by the heart. The integrity and elasticity of the vessels and the modulation of blood pressure are determined by smooth muscle cells and ECs lining the vascular walls and by their relationship with ECM. EMILIN1 is intimately associated with elastic fibers and microfibrils in blood vessels (Colombatti et al., 1985, 2000). EMILIN1 is implicated in elastogenesis and in maintenance of blood vascular cell morphology (Zanetti et al., 2004). *Emilin*1$^{-/-}$ mice display elevated systemic blood pressure independent of cardiac output without defects in vascular contractility or mechanical properties. They have significantly narrower arteries causing the hypertensive phenotype. EMILIN1 binds to proTGF-β1 prior to the cleavage of LAP and upstream of the furin convertases and prevents its processing (Zacchigna et al., 2006). The increased TGF-β in the absence of EMILIN1 as it occurs in *Emilin*1$^{-/-}$ mice leads to reduced vascular cell proliferation and results in narrower blood vessels and increased peripheral resistance, thereby causing hypertension. Also *Emilin*2$^{-/-}$ and *MMRN*2$^{-/-}$ mice mice display hypertensive phenotypes but the underlying mechanisms are different and do not affect proTGF-β1 processing (G. M. Bressan et al., unpublished data). While the pathogenetic mechanism of blood hypertension is largely explained by the interaction between the EMI domain and proTGF-β, EMILIN1 may also be involved in the hypertensive phenotype with other domains; for instance, in anchoring smooth muscle cells to elastic fibers or in the process of vessel assembly. In fact, in the media of arteries there are specific sites of interaction between smooth muscle cells and elastic fibers (Daga-Gordini et al., 1987). At these sites the cell basement membranes are interrupted and the cell surface is in direct contact with the periphery of the amorphous elastin core, a region enriched in fibrillin (Sakai et al., 1986) and EMILIN1 (Bressan et al., 1993). The finding that EMILIN1 gC1q is an adhesive substrate (see below) and provides anchorage and/or homeostatic control of cell proliferation (see below) suggests that it might also contribute to affect the cell number and the size of smooth muscle cells in arterial walls.

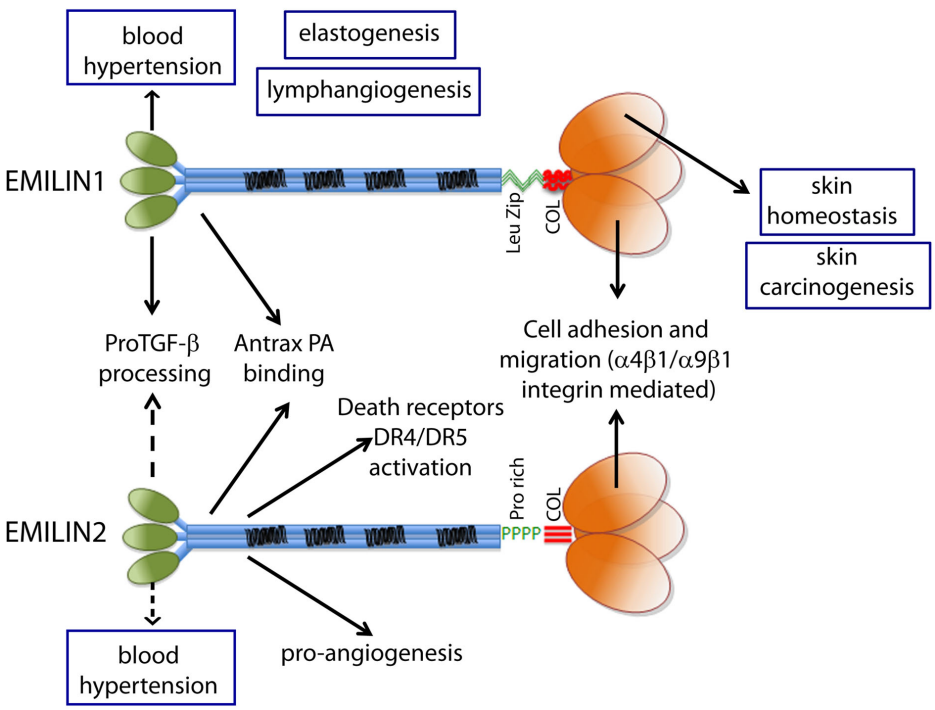


FIGURE 3 | The functions ascribed to the gC1q domain and to the other domains of EMILIN1 and EMILIN2 are summarized to show the versatility of these glycoproteins. The regulation of elastogenesis and of lymphangiogenesis have not yet been pinpointed to a specific domain although it is tempting to assume that gC1q plays an important role.

DEFENSE FUNCTION OF EMILIN/MULTIMERIN FAMILY MEMBERS

The informational spectrum method (ISM), a virtual spectroscopy approach, is a fast and simple structure analysis of proteins and their functionally important domains (Veljkovic and Metlas, 1988; Shepherd et al., 2003; Wen et al., 2005). By applying the ISM to the human sequence database all EMILIN/Multimerin family members were predicted to interact with anthrax protective antigen (PA). The interaction with PA was then formally proven for EMILIN1 and EMILIN2 by solid phase assays using recombinant proteins (Doliana et al., 2008). The interacting part of EMILIN2 resides in the first N-terminal 108 residues. Residue D425 of PA is important in mediating *Bacillus anthracis* cell toxicity (Ahuia et al., 2003) and its deletion results in a PA variant which still assembles but forms non-functional complexes leading to a complete inhibition of the channel forming ability of PA (Sellman et al., 2001). The deletion mutant (del425) of PA binds to EMILIN1 with very low efficiency suggesting that EMILIN1 interacts with this residue to prevent cell toxicity. The interaction is independent of the presence of divalent cations and does not involve PA aspartic residue at 683, a critical residue in cell surface receptor binding. The functional consequences of the specific interaction of PA *in vitro* and the inhibition of cell cytotoxicity by the LF–PA complex in the presence of EMILIN1 suggests that EMILINs/Multimerins, along with the LDL receptor-related protein LRP6 (Mayer et al., 2001), represent additional targets and/or binding proteins potentially useful for countermeasures against *B. anthracis* toxin lethality.

EMILIN2 AND CELL DEATH PROMOTION

Among the few ECM molecules that impair cell viability (Shichiri and Hirata, 2001; Todorovicç et al., 2005; Seidler et al., 2006; Ramakrishnan et al., 2007; Tai and Tang, 2008; Juric et al., 2009) EMILIN2 adopts an entirely different mechanism, i.e., the activation of the extrinsic apoptotic pathway through a direct binding to death receptor 4 (DR4) and 5 (DR5; Pan et al., 1997; LeBlanc and Ashkenazi, 2003). This binding, in analogy with the downstream events that occur after ligation of the death ligand TRAIL to DR4 or DR5, is followed by receptor clustering, co-localization with lipid rafts, DISC assembly, and caspase activation (Mongiat et al., 2007). Thus, EMILIN2 mimics the activity of the known death receptor ligands (Walczak et al., 1997; Ashkenazi, 2008; Johnstone et al., 2008). This pro-apoptotic function resides in a homotrimeric 90 residues long region toward the N-terminus of the molecule, positioned right after the EMI domain in the coiled-coil domain (Mongiat et al., 2010). Since normal cells do not express DR4 or DR5 it can be speculated that EMILIN2 or specific peptides encompassing the pro-apoptotic region might be used as anti-tumor agents in combination with anti-angiogenic drugs such as bevacizumab (Mongiat et al., 2010) and in cases where TRAIL resistance is involved (Hengartner, 2000; Fulda, 2009).

MMRN2, EMILIN2, AND ANGIOGENESIS

MMRN2 is found along the blood vessels in close contact with ECs in normal and tumor vasculature (Sanz-Moncasi et al., 1994; Christian et al., 2001; Koperek et al., 2007) and contributes to the maintenance of blood vessels' homeostasis by affecting the VEGF-A/VEGFR2 axis (Lorenzon et al., 2011). Neither proliferation nor apoptotic rate are affected. By preventing the interaction of VEGF-A with VEGFR1 and VEGFR2, MMRN2 reduces their activation and impaires the non-integrin dependent motility of ECs leading to a decreased intra-tumoral angiogenesis and consequent impaired tumor growth. It is conceivable that MMRN2 attains a high EC pericellular concentration and thus competes for the binding of VEGF-A to its receptors. MMRN2 may thus represent a novel key homeostatic molecule indispensable for the maintenance of blood vessel integrity.

Despite the strong reduction of tumor growth induced by the pro-apoptotic function of EMILIN2 (Mongiat et al., 2007, 2010), EMILIN2 also increases the overall intratumor blood vessel density. As a consequence, added bevacizumab is able to further inhibit tumor growth (Mongiat et al., 2010).

MMRN1 AND PLATELET FUNCTION

MMRN1 is a soluble S–S linked homopolymer stored in platelets, megakaryocytes, and ECs and deposited in ECM (Hayward, 1997; Adam et al., 2005). It supports the adhesion of platelets, neutrophils, and ECs via integrin $\alpha v\beta 3$ and $\alpha_{IIb}\beta 3$ (Adam et al., 2005). It binds to collagen and it is able to enhance von Willebrand factor-dependent platelet adhesion to collagen thus supporting thrombus formation. MMRN1 has a high affinity for factor V (Jeimi et al., 2008) and this facilitates the co-storage in platelet α-granules. When MMRN1 is released from platelets during platelet activation it regulates thrombin formation limiting thrombus formation. This protein is playing an important homeostatic control in platelets aggregation and its consequences. Unfortunately, all these multiple functions have not been assigned to any of the domains of MMRN1 apart from cell adhesion that depends on an RGD peptide at the N-terminus (Adam et al., 2005).

gC1q-DEPENDENT FUNCTIONS

HOMOTRIMER ASSOCIATION

Several members of the C1q/TNF superfamily associate into trimers. The chain composition of these different superfamily members varies from heterotrimers formed of three distinct chains as in C1q, or of homotrimers as in collagen types VIII (Rosenblum, 1996; Illidge et al., 1998; Greenhill et al., 2000), and X (Thomas et al., 1991; Reichenberger et al., 1992; Chan et al., 1996; Frischholtz et al., 1998), ACRP30 (Hu et al., 1996; Shapiro and Scherer, 1998), MMRN1 (Hayward et al., 1995). The potential self-interaction of the EMILIN1 gC1q domain was formally demonstrated with the use of two-hybrid system approaches, yeast mating and cotransformation that both confirm the self-association of the single protomers (Mongiat et al., 2000).

SUPRAMOLECULAR ASSEMBLY

The smallest EMILIN1 protomer under reducing conditions is a trimer held together by strong non-covalent bonds. Likely the formation of the gC1q homotrimer acts as a nucleation in a C- to N-terminal direction, facilitating the formation of the triple helix. Several of the gC1q domain-containing molecules assemble to high order S–S bonded structures composed of 9–12 polypeptides multimers, as in ACRP (Scherer et al., 1995), 18 polypeptides as in C1q (Reid, 1983; Kishore and Reid, 2000), collagens type X (Kwan et al., 1991), and probably also type VIII (Sawada et al., 1990). The sizes of MMRN1 multimers are well above 24 polypeptides (Hayward et al., 1991). EMILIN1 multimers can reach sizes of several

million daltons, corresponding to S–S bonded complexes composed of several dozens of polypeptides (Mongiat et al., 2000). We have preliminary evidence that secreted recombinant EMILIN2 is assembled in smaller multimers compared to EMILIN1.

At present, the mechanistic basis for the formation of EMILINs supramolecular aggregates is unknown. While covalent S–S bonds are involved in the association it is possible that also non-covalent interactions participate to allow supramolecular assembly. In addition, it is not clear whether each EMILIN exclusively forms homotypic macro-aggregates consisting of only one member or also heterotypic macro-aggregates consisting of more members. This latter possibility is supported, at least for EMILIN1 and EMILIN2, by the partial overlapping gene expression pattern *in vivo* in many tissues (Braghetta et al., 2002), and by the codistribution of the secreted deposited proteins in *in vitro* cell cultures (Doliana et al., 2001). In situations where both EMILINs are expressed simultaneously, it is theoretically possible that each EMILIN forms either separate homotypic or that they may form heterotypic assemblies. The isolation of EMILIN2 from expression libraries using the gC1q domain of EMILIN1 as a bite proved that gC1q domains from distinct but similar molecules could interact at least in the two-hybrid system (Doliana et al., 2001). Then, also homo- and hetero-interactions between gC1q and EMI domains were determined by qualitative and quantitative two-hybrid assays and by immunochemical evidence of co-expression in yeast human cell extracts (Bot et al., in preparation). It is possible, at least *in vitro*, that gC1q and EMI domains from the same or different EMILINs interact in a head-to-tail fashion. The question remains whether there is the need for adapter molecules that favor this interaction or whether this interaction is direct and EMILIN1 and EMILIN2 can co-polymerize head-to-tail to form heterotypic non-covalent multimers *in vivo*.

INTERACTION OF gC1q WITH α4β1 INTEGRIN

α4β1 integrin binds to the cell surface vascular cell adhesion molecule-1 (VCAM-1) on activated endothelium (Hamann et al., 1994) and to the ECM molecule fibronectin (FN; Humphries et al., 1986; Komoriya et al., 1991). The key VCAM-1 binding site is the tripeptide motif sequence IDS. This sequence is homologous to the LDV active motif on the CS-1 peptide of FN, suggesting that α4β1 may interact with FN and VCAM-1 through a similar mechanism. EMILIN1 is an adhesive ligand for α4β1 (Spessotto et al., 2003, 2006; Verdone et al., 2008; Danussi et al., 2011) and this function is fully accounted for by the gC1q domain (Spessotto et al., 2003). All polypeptides with integrin binding capability display exposed aspartic or glutamic acid residues located in mobile loops protruding from the main core of the ligand (Leahy et al., 1996; Casasnovas et al., 1998). These residues are critical for integrin recognition. The three dimensional conformation of the active site on ligand–receptor binding is extensively demonstrated (in particular for RGD containing ligands; Humphries, 1990; Leahy et al., 1996; Arnaout et al., 2007; Barczyk et al., 2010). The aspartic-acid-based sequences (e.g., RGD, LDV, KGD, RTD, and KQAGD) bind to the majority of integrins (Yamada, 1991) and mutations around the aspartic acid residue, i.e., G in RGD or L in LDV, greatly affect integrin recognition (Pierschbacher and Ruoslahti, 1984; Komoriya et al., 1991; Cherny et al., 1993). Other integrins interact with ligands that contain glutamic-acid-based sequences (Michishita et al., 1993). However, it is likely that the scenario in the EMILIN1–α4β1 interaction is more complex than in conventional α4β1 binding to short linear peptide consensus sequences, representing the common feature of ECM integrin ligands (Humphries, 1990; Humphries et al., 2006). A gC1q domain in which the glutamic acid at position 933 (E933) was substituted with an alanine residue is no longer functional in cell adhesion assays and it is not recognized by α4β1 integrin suggesting that this residue plays a major role in this interaction. We exclude that the loss of activity of the E933A mutant or of deletion mutants encompassing this residue is due to a global folding change of the protein (Verdone et al., 2008). Thus, the gC1q–α4β1 interaction provides a further example of the functional relevance of disordered regions in proteins (Radivojac et al., 2007).

How would an homotrimeric molecule such as gC1q mechanistically interact with the α4β1 integrin? The explanation that a linear tripeptide from one single monomer as in CS-1 peptide of FN could represent the α4β1 ligand binding site on gC1q is apparently excluded by the finding that the L932A mutant does not loose its cell adhesion binding property. Several mutant residues around E933 are still functional and this indicates that the α4β1 integrin binding site involves only E933. Thus, this residue plays an absolutely central role in α4 integrin mediated interaction, whereas several residues close to E933 do not seem to participate in this interaction (Verdone et al., 2008). The NMR solved structure locates residue E933 in the flexible loop at the apex of the EMILIN1 gC1q domain assembled into a symmetric trimer. The stoichiometry of three integrin molecules each recognizing one E933 residue on the unstructured loops of the gC1q trimer is ruled out for steric reasons. Thus, the interaction pattern with α4β1 might well entail the binding of the three E933 carboxylate groups to a single integrin molecule. An example of an integrin–ligand interaction that requires two structural elements residing on different polypeptide chains has been already reported: to be recognized by αvβ3 integrin the LG1–3 globular domain of laminin α chain requires the intact heterotrimeric C-terminal portion of the coiled-coil domain, including the most distant C-terminal region of the β and γ chains of laminin (Künneken et al., 2004). In that case it has been hypothesized that the C-terminus of the γ chain is assembled together with a specific region of LG1–3, leading to the formation of an interchain functional ligand tripeptide. The gC1q–α4 interaction could represent the first example of an integrin binding site located on a homotrimeric assembly that employs a different mode of integrin engagement and prospects a new model for the interaction geometry recognition between an integrin binding site and a functional ligand located on a homotrimeric assembly. In this scenario we hypothesize the involvement of two/three E933 residues for a single integrin molecule engagement for a proper interaction able to fit the α4 pocket in the βMIDAS region. One possibility to tackle this question is the use of bicistronic vectors coding for wild type and non-functional mutant E933A gC1q in order to obtain trimers with a single copy or two copies of the wild type sequence on average in the homotrimer. In this systems the mutant and wild type monomers are randomly assembled into trimers to form a heterogeneous population and can be isolated by affinity chromatography. Preliminary data suggests that

cell adhesion to heterogeneous gC1q trimers composed by both wild type and E933A mutants, is negatively affected implying the need of all three wild type monomers to achieve a proper integrin ligation with the homotrimer (Capuano et al., in preparation).

FUNCTIONAL CONSEQUENCES OF THE gC1q/INTEGRIN LIGATION

CELL ADHESION

It is known that the adhesion of α5β1 or αvβ3 integrins to FN and vitronectin (VN) promotes a profound cytoskeletal reorganization (Ruoslahti and Pierschbacher, 1987; Defilippi et al., 1999; Ruoslahti, 1999; Zamir and Geiger, 2001; Campbell, 2008). On the contrary, the α4β1-dependent interactions extensively studied in hematopoietic cells (Morimoto et al., 1998; Steeber et al., 2005; Rose et al., 2007) as well as in transfected adherent cells (Pinco et al., 2002) prompt only the initial and intermediate stages of cell adhesion, i.e., attachment and spreading, whereas focal adhesion and stress fiber formation, characterizing strong cell adhesion, are rarely if ever observed (Iida et al., 1995).

Contrary to common beliefs that consider α4β1 an exclusive leukocyte integrin, there is a wealth of data available that demonstrates that this integrin is widely expressed, although at lower levels, in normal tissues including brain, heart, kidney, lung, muscle, liver prostate, skin, and ovary as well as their tumoral counterparts; α4β1 is also expressed in colon, bladder, breast, cervix, and melanoma tumor cells (Holzmann et al., 1998). In addition, its expression is upregulated by inflammatory cytokines (Weber et al., 1996; Milner and Campbell, 2003). This data makes α4β1 an integrin which is potentially involved in multiple and extensive interactions under several biological conditions including tumor spread.

Cells attached to EMILIN1 display a pattern of actin and paxillin distribution with accumulation of ruffles-inducing signals and lack of polarization and of stress fiber formation (Spessotto et al., 2003). Recognition of the gC1q domain does not require exogenous activation of α4β1 nor the addition of Mn^{2+} nor anti-β1-activating mAbs; instead the presence of Ca^{2+} or Mg^{2+} is necessary since EDTA treatment fully abolishes cell adhesion (Spessotto et al., 2003). Similar results are observed upon binding to EMILIN2 (Spessotto et al., unpublished observations). On the contrary, MMRN1 that possesses a RGD site binds to αvβ3 and $\alpha_{IIb}\beta3$ integrins on platelets (Adam et al., 2005).

CELL MIGRATION

Among the numerous cell types that migrate on EMILIN1 trophoblasts are particularly interesting. The finding that trophoblast cells could attach to and very efficiently migrate and haptoractically move toward EMILIN1 using the α4β1 integrin without any prior artificial cellular activation was rather unexpected (Spessotto et al., 2006) because directional haptotaxis was much more efficient toward EMILIN1 compared with FN. In addition, under several conditions the cooperation of MMPs with integrins plays a contributing role in order to better direct cellular migration (Deryugina et al., 2001). Accordingly, MT1-MMP is strongly upregulated when trophoblasts cells are co-cultured with EMILIN1-producing decidual stromal cells. The finding that the general MMP inhibitor GM6001 is more effective and affects cell migration toward EMILIN1 at 1-h migration, whereas at 4 h the inhibitory effect is stronger when cells move in an α5β1 integrin dependent mode toward FN (Spessotto et al., 2006), suggests that both ligands might be involved in the uterine wall invasion process but with a sequential role. Deposited EMILIN1 in the uterine tissue could thus favor the initial α4β1 dependent invasion by trophoblast cells to be assisted at later times by other ECM constituents such as FN and different integrins.

CELL PROLIFERATION

It is generally known that integrin engagement positively regulates cell growth (Clark and Brugge, 1995; Walker and Assoian, 2005; Gilcrease, 2007; Streuli, 2009). The finding that EMILIN1 by the direct engagement of the gC1q domain modulates skin cell proliferation points out a novel function of α4β1 as well as the structurally and functionally similar α9β1 integrin (Danussi et al., 2011). The lack of integrin occupancy by EMILIN1, as it occurs in *Emilin*$1^{-/-}$ mice, results in increased Ki67 positive cells in epidermis and dermis. This is accompanied by reduction of PTEN phosphatase and strong upregulation of pErk1/2. Accordingly, when HT1080 and CaCo-2 cells expressing α4β1 or α9β1 integrins, respectively, adhere to gC1q PTEN levels increase. Even the addition of soluble gC1q to cells attached to plastic upregulates PTEN and the addition of TGF-β further increases PTEN levels. This is accompanied by decreased pErk1/2 levels and suggests a link between PTEN and α4/α9 integrin engagement: when α4/α9 integrins are not ligated by gC1q PTEN is down-regulated determining the activation of proliferative pathways such as pAkt and pErk1/2. Mechanistically, the increased pErk1/2 reduces TGF-β signaling by the phosphorylation of Smad2 at inhibitory Ser245/250/255 residues. It seems likely that these downstream changes concur *in vivo* to the decreased proliferation of basal keratinocytes and dermal fibroblasts (**Figure 4**).

ROLE OF EMILIN1 gC1q IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

Targeted inactivation of the *Emilin*1 gene in the mouse induces multiple phenotypes characterized by: decreased diameter of arterial vessels and systemic hypertension (Zacchigna et al., 2006); a lymphatic phenotype with a significant reduction of anchoring filaments (AFs) and enlargement of lymphatic vessels leading to a mild lymphatic dysfunction (Danussi et al., 2008); dermal and epidermal hyperproliferation (Danussi et al., 2011). Only the last phenotype is strictly dependent on the lack of gC1q.

EMILIN1 IN LYMPHATIC VESSEL FUNCTION

The connections between ECM and lymphatic endothelial cells (LECs) that critically affect lymphatic vasculature function are mediated by AFs (Swartz and Skobe, 2001). These fibrillin rich structures, which are part of a hierarchical fibrillar elastic apparatus (Gerli et al., 2000), are highly sensitive to interstitial stresses (Swartz and Skobe, 2001) and exert a significant tension on LECs: their main function is to widen the capillary lumen and open the overlapping cell junctions facilitating lymph formation (Cueni and Detmar, 2006). Abnormalities of AFs may reduce adsorption from interstitium and propulsion of lymph and cells and promote pathological conditions such as lymphedema or diseases related to

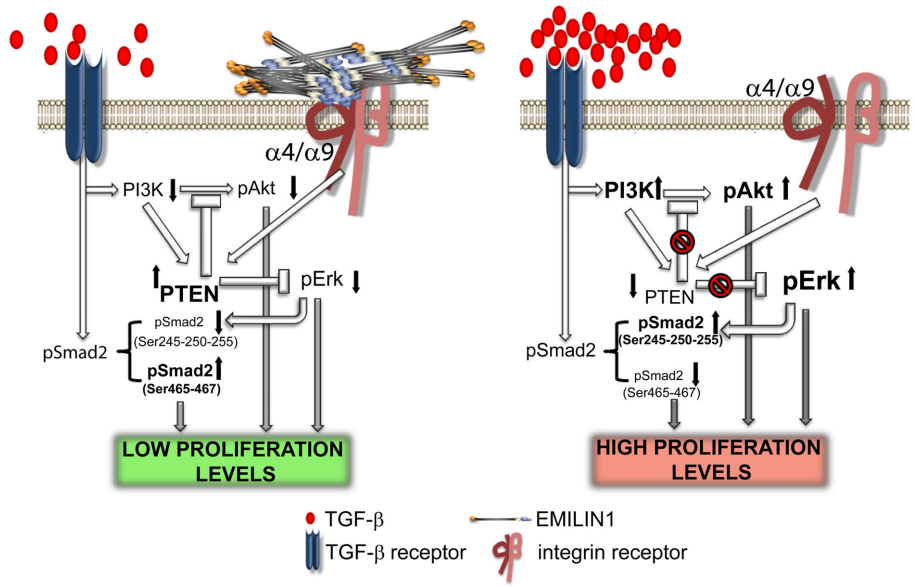


FIGURE 4 | Elastin microfibrillar interface protein 1 in skin homeostasis. This illustration summarizes the proposed molecular mechanism underlying the regulatory role of EMILIN1 in skin. When EMILIN1 is expressed (left) TGF-β triggers cytostatic signals via pSmad2 (Ser465/467), down-modulates PI3K/Akt and hence PTEN expression is increased also because EMILIN1 binds to α4/α9β1 integrins. PTEN activation then inhibits the pro-proliferative activity of pErk1/2. When EMILIN1 is not present (right) the increased levels of mature TGF-β and the lack of α4/α9β1 integrin specific engagement downregulates PTEN determining the activation of the pro-proliferative pAkt and pErk1/2 pathways. In addition, TGF-β signaling is reduced since Erk1/2-phosphorylates the inhibitory Ser245/250/255 residues of Smad2.

impaired immune responses (De Cock et al., 2006). Dysfunctions may also be induced by extensive and chronic degradation of the ECM that renders lymphatic vessels non-responsive to the changes in the interstitium (Pelosi et al., 2007).

Consistent with the absence of a basement membrane *in vivo* (Pepper and Skobe, 2003), LECs secrete very little ECM (Farnsworth et al., 2006). EMILIN1 is produced by LECs *in vitro* and is present at the abluminal side of lymphatic vessels. It forms fibers directed from LECs to the surrounding ECM. Therefore, the detection of abundant EMILIN1 suggested the involvement of this protein in the structure and function of lymphatic vessels (Danussi et al., 2008). In fact, a comparative study between wild type and *Emilin*1$^{-/-}$ mice highlighted that *Emilin*1$^{-/-}$ mice display hyperplasia and enlargement of lymphatic vessels compared to wt mice. Morphological alterations of the lymphatic vessels of *Emilin*1$^{-/-}$ mice also are evident at the ultrastructural level. Structural abnormalities such as the reduction in the number of AFs and the formation of extensive multiple overlapping junctions of LECs are among the most evident alterations. LECs in *Emilin*1$^{-/-}$ mice frequently appear detached from the underlying ECM and from each other and result in the formation of irregular and multiple intraluminal flaps suggesting that EMILIN1 is involved in the process of lymphatic remodeling observed in these mice (Danussi et al., 2008). These alterations are associated with inefficient lymph drainage, enhanced lymph leakage, and lymphedema. However, in contrast to other recently described lymphatic-lineage gene targeting in mouse models, that in most cases result embryonic or perinatal lethal (Tammela et al., 2005), homozygous disruption of *Emilin*1 gene induces only a mild phenotype. Notably, this is the first abnormal lymphatic phenotype associated with the deficiency of an ECM protein. Finally, the valve defects observed in collecting vessels are grossly similar to those of α9 integrin null mice (Bazigou et al., 2009), suggesting that the interaction of EMILIN1 and α9 integrin demonstrated with basal skin keratinocytes (Danussi et al., 2011) may also be important in valve morphogenesis in LECs.

EMILIN1 IN SKIN HOMEOSTASIS

The phenotype of the *Emilin*1$^{-/-}$ mouse skin revealed increased thickness of epidermis and dermis.

Elastin microfibrillar interface protein 1 interacts with α4β1 (expressed on fibroblasts) and the closely related α9β1 (expressed on keratinocytes) to provide an important external cue for the maintenance of a correct homeostasis between proliferation and differentiation (Danussi et al., 2011). In this context, signals emanating from EMILIN1-ligated α4/α9 integrins are anti-proliferative (Danussi et al., 2008, 2011). But how does EMILIN1 brings about the control of cell proliferation? EMILIN1 is produced only by skin fibroblasts and it is deposited in the dermis where it is abundantly expressed. However, EMILIN1-positive fibrils depart from near the BM and reach the basal keratinocyte layer, thus connecting the epidermis to the underlying dermal layer. *In vitro* co-cultures of EMILIN1-producing fibroblasts and keratinocytes provided the formal demonstration that the contact between deposited EMILIN1 and α4/α9 integrins directly regulates cell proliferation: only "contact" and not "transwell" (so that the two cell types are physically separated) co-cultures of keratinocytes with wild type EMILIN1-producing fibroblasts

inhibits proliferation. Consequently, silencing of EMILIN1 stimulates the proliferation of co-cultured keratinocytes. Proliferative and anti-proliferative signals occur simultaneously and the latter, EMILIN1/gC1q ligation in this case, can override the pro-proliferative signals only when they reach a certain threshold (Müller et al., 2008). It is likely that under certain circumstances the pericellular concentration of EMILIN1 in basal keratinocytes (and fibroblasts) exceeds this threshold and stops cell proliferation. Our findings open new perspectives in the molecular mechanisms of quiescence versus proliferation in basal keratinocytes. In addition, given the high expression of α9β1 integrin on these cells, the demonstration that EMILIN1 is a novel ligand for this integrin (Danussi et al., 2011) supports a scenario in which one of the functional consequences is the integration of EMILIN1 into the complex connections of basal keratinocyte turn over and the cross talks between basal keratinocytes, underlying ECM and stromal cells.

EMILIN1 IN CANCER DEVELOPMENT

In *Emilin*1$^{-/-}$ mice subjected to a skin carcinogenesis protocol tumor appearance was significantly accelerated and the number and size of tumors compared to wild type mice increased (Danussi et al., submitted). It seems likely that the aberrant skin homeostasis generated by EMILIN1 deficiency in these mice (Danussi et al., 2011) induced a pro-tumorigenic environment. Functional studies support the hypothesis that PTEN is a critical tumor suppressor for skin cancer in humans and in mice (Di et al., 1998; Segrelles et al., 2002; Suzuki et al., 2003; Komazawa et al., 2004) by negatively regulating signal pathways involved in cell proliferation (Schindler et al., 2009). In *Emilin*1$^{-/-}$ mice the lack of EMILIN1 ligation of α4β1/α9β1 integrins reduces the expression of PTEN also in the skin tumor environment. One attractive hypothesis assumes that the mechanisms controlling the homeostasis of cell proliferation and hence the enhanced tumor development when EMILIN1 expression is genetically or functionally knocked down depends on the unique mode of gC1q–α4β1/α9β1 integrin interaction.

We are confident that the elucidation of the fine molecular interactions between α4β1/α9β1 integrins and the E933 residue(s) on the unstructured loops of the gC1q trimer and the downstream signaling events will help clarify the numerous gC1q-dependent cellular responses.

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Emerging and novel functions of complement protein C1q

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Complement protein C1q, the recognition molecule of the classical pathway, performs a diverse range of complement and non-complement functions. It can bind various ligands derived from self, non-self, and altered self and modulate the functions of immune and non-immune cells including dendritic cells and microglia. C1q involvement in the clearance of apoptotic cells and subsequent B cell tolerance is more established now. Recent evidence appears to suggest that C1q plays an important role in pregnancy where its deficiency and dysregulation can have adverse effects, leading to preeclampsia, missed abortion, miscarriage or spontaneous loss, and various infections. C1q is also produced locally in the central nervous system, and has a protective role against pathogens and possible inflammatory functions while interacting with aggregated proteins leading to neurodegenerative diseases. C1q role in synaptic pruning, and thus CNS development, its anti-cancer effects as an immune surveillance molecule, and possibly in aging are currently areas of extensive research.

Keywords: complement, C1q, apoptosis, neurogenesis, pregnancy, cancer

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Introduction

C1q, the first recognition subcomponent of the complement classical pathway, is a 460 kDa hexameric glycoprotein (1–3), which is composed of 18 polypeptide chains, composed of 9 non-covalently linked subunits having 6 of A (223 residues; 34 kDa), B (226 residues; 32 kDa), and C (217 residues; 27 kDa) chains (**Figure 1**). These chains have a short N-terminal region (3–9 residues), a collagen region having 81 residues and a C-terminal globular (gC1q) domain of about 185 residues (3, 4). The hexameric C1q molecule has a tulip-like structure made up of structural units, which combine in the fibril-like central portion (5). Crystal structure of the heterotrimeric gC1q domain, solved at 1.9 Å resolution, revealed an almost spherical (diameter of 50 Å), dense hetrotrimer associated by non-polar forces with Ca^{2+} ion bound at the top, showing a classical jellyroll topology (6). A strong structural homology with gC1q domain [mouse ACRP30, human collagen X, mouse collagen VIII

Abbreviations: CERT, ceramide transporter protein; CJD, Creutzfeldt–Jakob disease; CNS, central nervous system; CRP, C-reactive protein; DAF, decay-accelerating factor; DC, dendritic cells; dsDNA, double-stranded DNA; FDC, follicular dendritic cells; GABA, gamma-aminobutyric acid; HIV, human immunodeficiency virus; IL-6, interleukin-6; HTLV-1, human T-lymphotropic virus 1; LAIR, leukocyte-associated Ig-like receptor; LPS, lipopolysaccharides; MAC, membrane attack complex; MCP, membrane co-factor protein; NGF, nerve growth factor; pDC, plasmacytoid dendritic cell; PrP, prion protein; PrPSc, scrapie-associated prion protein; PS, phosphatidylserine; RGC, retinal ganglionic cells; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; TNF-α, tumor necrosis factor-alpha; TGF-β, transforming growth factor-beta.

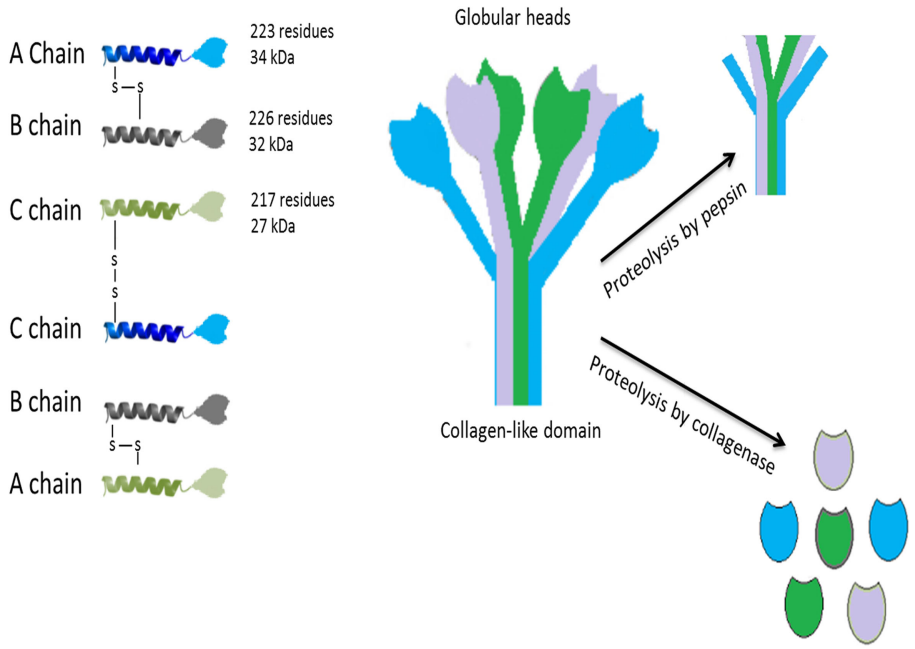

FIGURE 1 | Structure of C1q. The hexameric C1q molecule (460 kDa) has a tulip-like structure, composed of nine non-covalently linked subunits of six A, B, and C chains; A–B dimer is 52,750 Da and C–C dimer is 47,600 Da. Fragments of C1q after pepsin digestion at pH 4.4 contain six intact connecting strands and small non-collagen-like peptides. After partial proteolysis with collagenase at pH 7.4, the globular regions (gC1q) appear intact. C1q binds to Fc regions of IgG or IgM, HIV-1, phosphatidylserine, HTLV-1, C-reactive protein, damaged neurons, myelin debris, amyloid fibrils, and apoptotic cells via the gC1q domain. The collagen-like region binds to serine proteases such as C1r and C1s leading to activation of the classical pathway. Collagen region of C1q also binds to C1q receptors (most notably, cC1qR or calreticulin using CD91 as a cell-surface adaptor) to augment phagocytosis by phagocytic cells and mount a pro-inflammatory immune response.

(α1) and human C1q] has given rise to a C1q family with a β sandwich jellyroll topology similar to tumor necrosis factor (TNF), and hence, a C1q–TNF superfamily (7, 8).

C1q is a pattern recognition molecule as it has the ability to identify various structures and ligands on microbial surfaces, apoptotic cells, or indirectly via antibodies and C-reactive protein (CRP). C1q can bind IgG, IgM, HIV-1, phosphatidylserine (PS), HTLV-1, CRP, and many other ligands via gC1q domain, and also to various receptors including calreticulin-CD91, and integrin $\alpha_2\beta_1$ (4, 9). Binding of C1q to most of its ligands is via gC1q domain, which involves recognition of charged patterns/clusters (7). Recently, a number of functions of C1q have been described that do not involve classical pathway activation, suggesting that C1q has an additional role to play in homeostasis and development.

Modulation of Dendritic Cells Maturation and Functions by C1q

Dendritic cells (DCs) are antigen capturing and presenting cells of innate immunity (10, 11). Interstitial/dermal DCs (IDDCs) produce significantly more C1q at 48 h, following stimulation with IL-4 and granulocyte macrophage-colony stimulating factor (GM-CSF), as compared to Langerhans cells (12, 13). C1q has been shown to be produced by renal DCs in a mouse model of progressive lupus nephritis. Renal sections of mice showed significantly higher expression of C1q in tubulo-interstitium of old MRL/lpr mice as compared to young MRL/lpr mice. Increased co-localization of MHC-II$^+$ cells with C1q was detected in old MRL/lpr mice (5 months) with lupus nephritis. Production of C1q derived from bone marrow precursors showed expression of C1q similar to other local sources such as macrophages. Furthermore, human kidney biopsies of systemic lupus erythematosus (SLE) patients showed an increased production of C1q by renal DCs (10).

C1q binds monocyte-derived immature DCs causing the NF-κB nuclear translocation from the cytoplasm. C1q enhances maturation of DC exhibiting raised surface expression of CD83, CD86, HLA-DR, and CCR7. Both gC1q and collagen region can independently induce DC maturation. In the presence of C1q, DCs secrete more IL-12p70 than immature DCs (14). C1q-primed mature DCs induce production of IFN-γ by co-cultured T lymphocytes (Th1 cells), stimulated by IL-12, and secreted by C1q-treated mature DCs (14). C1q produced by DCs is capable of activating complement and binding to apoptotic cells. C1q production by DCs is suppressed by IFN-α (15). Interestingly, C1q inhibits IFN-α production by plasmocytoid DCs (pDCs) induced by immune complexes (ICs), but enhances IFN-α production induced by herpes simplex virus (16).

Leukocyte-associated Ig-like receptor (LAIR; a transmembrane protein and a member of immunoglobulin super family) (17) is expressed on NK cells, B cells, monocytes, human fetal thymocytes, and T cells. LAIR 1 is an inhibitory receptor, which is considered to be involved in down-regulation of immune response

(17). C1q binding to LAIR leads to the activation of LAIR-1 signaling. C1q inhibits the GM-CSF and IL-4 stimulated differentiation of monocytes into DCs. Interaction of C1q with LAIR-2 reduces the expression of LAIR-1 and CD14, implying that the C1q-mediated differentiation of DCs is reversed by LAIR-2 (18). C1q and LAIR-1 also function to inhibit IFN-α production by plasmacytoid DCs of either healthy or SLE individuals. This suggests that C1q and LAIR-1 interaction is involved in the arrest of monocyte differentiating to DCs, and in the inhibition of plasmacytoid DC activation (18). Furthermore, C1q expression by DCs can be localized at the arterial wall. Atherosclerotic lesions showed intracellular and extracellular expression of C1q, particularly the necrotic core surroundings were C1q-positive. C1q expression was not only limited to DCs but also to other cell types such as macrophages, neovascular endothelial cells within atherosclerotic plaques, endothelium of vasa vasorum in the adventitia, and in very few luminal endothelial cells. The expression of DCs in the arterial lesions was heterogeneous, implying that some DCs may reside in the arterial wall for local synthesis of C1q (19). Consistent with this, C1q binding to apoptotic cells enhances the phagocytosis and stimulates the production of IL-6, IL-10, and TNF-α by DCs, without affecting IL-12p70 production.

C1q binds to resting as well as activated B cells. C1q may be involved in the stimulation of B cells to either produce IgM or IgG isotypes in normal individuals or those with impaired B cell activity (20). Studies on C1q deficient$^{(-/-)}$ mice have demonstrated that positive selection of B cells and IgM autoantibodies is increased by intracellular self-antigens that are displayed by dying cells, and the negative selection of B cells is decreased. The positive selection by self-antigens may be involved in the IgM and C1q-dependent clearance of dying cells, limiting the contact of conventional B cells to immunogenic self-antigens (21). C1q deficient mice developed splenic hypercellularity, displaying signs of accelerated $CD4^+$ T cell activation, enhanced splenic plasma cells and total serum IgM levels. This suggests splenic monocytosis is caused by C1q deficiency as well as the activation of T cells in an autoimmune-prone mouse strain (22). C1q deficient mice also develop lupus-like disease and the apoptotic cells have elevated levels of autoantigens as these are not cleared by complement. Mice genetically deficient in C1q displayed high levels of IgM and IgG3 (23).

Clearance of Apoptotic Cells by C1q and Its Role in Immune Tolerance

Waste Disposal and Self-Tolerance Hypotheses

Apoptosis plays an integral part in development and homeostasis, through tissue and organ remodeling and in homeostatic cell turnover (24–26). C1q is important in development, and has a major role in clearing apoptotic cells, which may otherwise encourage autoimmunity. C1q deficiency predisposes one with a high risk to develop SLE, a prototype of autoimmune inflammatory disease (27). Two main hypotheses on C1q related autoimmunity have been proposed, being apparently complementary. In the first "waste disposal hypothesis," due to C1q deficiency in clearing apoptotic bodies, an accumulated bulk of altered self apoptotic neo-antigens may induce in lymphatic tissues an uncontrolled affinity maturation and IgG isotype switching of specific self-reactive B cell clones in a T cell dependent manner (27). According to the second "self-tolerance hypothesis," C1q and C4 also maintain peripheral tolerance-anergy of naturally occurring IgM producing self-reactive B cell clones against specific apoptotic antigens such as histone and dsDNA as part of apoptotic cell homeostasis. Some of these clones may be eliminated through their negative selection centrally in bone marrow and spleen (28). Nuclear antigens are highly conserved and may cross-react with bacteria. Their autoimmunity is typical of SLE (29, 30).

Apoptosis is an evolutionarily preserved death program mediated by caspase proteases and regulated by pro-apoptotic and anti-apoptotic signals of adjacent cells (24). Affected cells undergo characteristic cell shrinkage, non-random nuclear protein and DNA fragmentation, plasma membrane blebbing, and exposure of PS on their membrane surface. Blebs-apoptotic bodies are rich in nuclear material including dsDNA targeted by autoantibodies in SLE (24). PS is an important early apoptotic molecule recognized by C1q (in addition to other target ligands as shown in **Table 1**) and by other phagocytic cell receptors, such as CD36 and TIM-4. It enables a rapid cell phagocytosis before their release of inflammatory molecules (31, 32).

SLE is strongly associated with C1q and C4 deficiency. Hereditary C1q deficiency, which is very rare, confers in homozygous persons at an early age, a more than 90% risk of developing the disease (27, 56). Deficiency in C4 carries a disease risk of around 80%. However, SLE is a heterogeneous complex disease due to acquired C1q deficiency, although there may be remissions (57). Symptoms, which are very variable, include skin rash, light sensitivity, glomerulonephritis, and arterial-venous thrombosis. SLE is mediated by autoantibodies to various self-antigens, predominantly to C1q and apoptotic blebs containing nuclear antigens. Its hallmark is deposition of ICs with such antigens (58). Immunological changes can precede the disease, sometimes several years in advance (28).

C1q, C4, and C3 proteins are involved in specific antigen presentation by DCs to T and B cells. DCs are central in processing self and non-self-antigens during immune response (59). In a physiological steady state, tissue immature migrating DCs sample microbe and tissue antigens, and in draining lymph nodes differentiate into follicular DCs, where they mature and present processed specific antigens to naive T cells in a MHC-dependent manner. Such antigen specific Th cells, in turn, interact via their receptor with antigen specific B cell, which may require T cell help. FDCs can also directly present antigens to specific immature B cells in lymphoid cell compartment (29, 60). However, in most cases, for efficient antigen B cell receptor (BCR) activation, self or microbial antigens are covalently tagged with adjuvants C3d or C3dg fragments as opsonins (61).

Possible Involvement of C1q Receptors in Tolerance

Of a number of candidate C1q receptors, two receptors for C1q, cC1qR and gC1qR, have been largely documented in the literature. cC1qR (now known as calreticulin) was identified on phagocytic cell surface as a receptor for the collagen region of C1q (and collectins) (62). Another C1q receptor specific to the

TABLE 1 | Emerging non-complement functions of C1q.

C1q functions	Mechanisms	Ligands/receptors	Reference
Canonical Wnt signaling, induces aging	C1s-dependent cleavage of the ectodomain of LRP6	Frizzled receptors (Fz) LRP5/6	(33)
Invasion of epithelial and endothelial cells	C1q collagen region with the host cell-surface receptor interaction	*S. pneumoniae* serotype 35A (NCTC10319), *S. pneumoniae* strain D39 (serotype 2)	(34)
Clearance of bacterial pathogens	Gram-positive (*S. pyogenes*) and Gram-negative (*E. coli*)	Collagen region; outer membrane protein from Gram −ve bacteria Lipopolysaccaride, fibronectin, fibrin, fibrinogen	(34–36)
Escape mechanism for *Streptococcus pneumoniae*	C1q and PepO interaction	Pneumococcal endopeptidase O (PepO)	(37)
Induction of apoptosis in cells overexpressing WOX1	Phosphorylation of N-terminal WW domain of WOX1	WOX1	(38)
Induction of angiogenesis	Vessel formation during wound healing	gC1q domain	(39)
Clearance of apoptotic cells	gC1q domain	PS dsDNA Calreticulin Annexin A2 and A5	(40–44)
Tolerance induction	Immature DCs	C1q/gC1qR cC1qR	(45, 46)
Developmental synaptic pruning	Microglia		(47)
Uptake and transmission of PrP^{Sc} from the gut to the brain	Conventional DCs	Calreticulin	(49, 50)
Forms complexes of PrP oligomers		gC1q domain	(51)
Development of DC	DC	gC1qR	(52)
Induction of tolerance phenotype Tolerogenic effect (TGF-β1 release)		cC1qR	(53)
Negatively influences BCR signaling; enhances tolerance	B cell		(54)
Induces anti-inflammatory response	B cells		(55)
Inhibits activation and proliferation	T cell	gC1qR	(54)

globular head region, gC1qR, has also been described (63). Like C1q, the two receptors also seem to have multiple specificity and functions. C1q receptors are C1q collagen-like domain binding calreticulin (cC1qR), and C1q globular domain binding protein receptor (gC1qR) p33 (63). Calreticulin is part of multi-protein-binding scavenger receptor complex CD91, of which β chain is a transmembrane signaling protein. C1q/cC1qR/CD91 complex bound apoptotic cells can be taken up by immature DCs through their micropinocytosis (42, 43). Curiously, CD91 can also directly bind C1q (65). gC1qR is also an ubiquitous co-receptor with multi-ligand interaction and effector properties in various tissues. For example, in immature DCs, it interacts with transmembrane DC-SIGN-1 lectin receptor (66).

C1q, through two of its receptors (cC1qR and gC1qR), is involved differentially in monocyte–DC development (52). C1q may thus be involved in modulation of a specific antigen-associated phenotype of DCs, such as tolerance/anergy in the case of apoptotic nuclear antigens (63). Upon these antigen stimulations, mature DC subsets express immunosuppressive cytokines dominated by TGF-β and IL-10 (67), which may impact on specific self-reactive B cell clone development (30). Moreover, tissue migrating DC subsets, in contrast to resident follicular DC derived from blood, have the capacity to transform $CD4^+$ Th cells into peripheral $CD4^+$ $CD25^+$ FoxP3 T-regulatory cells (Tregs), specific for an antigen. For example, myelin specific Tregs strongly ameliorated experimental autoimmune encephalomyelitis (EAE) (68). A subpopulation of monocytes migrating from various tissues to lymph nodes, express only minimally DC markers, while ingesting antigens (69). Such DC apoptotic and other antigen specific tolerogenic bias are apparently impaired in C1q deficiency, which may result in autoimmunity.

C1q, as a molecular pattern sensor, can directly bind PS as well as to nuclear antigens including dsDNA exposed on apoptotic blebs/bodies of various tissue cells such as keratinocytes and vascular endothelial cells. These complexes may be rapidly taken up through its C1q receptors by non- and professional phagocytes such as immature DCs and macrophages (40, 70). This feature has been studied with UV-B irradiated early apoptotic HeLa cells. C1q strongly interacted via multiple regions of its gC1q domain with PS on apoptotic cells (41). Calreticulin binds to gC1q, as well as to C1q collagen-like domain (cC1q) (71, 72). gC1q domain also possesses a lectin-like region in recognizing late apoptotic bodies DNA, i.e., its deoxy-D-ribose form (73).

cC1qR and gC1qR contribute to the development of DCs, during which they become differentially expressed, and consequently to naïve T-cell antigen presentation and to antigen specific T cell–B cell interaction (52). Normally, a steady state relation between both C1q receptors may be maintained, which may be perturbed in C1q deficiency. DCs are major extrahepatic producers of C1 complex, which can become expressed on their surface, acting in a paracrine way. This may include facilitating antigen presentation to naïve T cells (74). Such anchored C1q might on

contact with apoptotic cell, help to induce in DCs a tolerogenic phenotype. As studied with macrophages, an initial close contact with apoptotic cells, possibly by tethering receptors, is needed for these tolerogenic effects, including TGF-β1 release. This cell state appears to be independent of receptors, which mediate PS exposed apoptotic cell phagocytosis such as CD36 and $\alpha_v\beta_5$ integrin, as tested with mice deficient in these genes (53).

TIM and Tolerogenic Phenotypes

A PS-specific apoptotic cell phagocyte receptor, TIM4 (T-cell immunoglobulin- and mucin-domain-containing molecule) has been cloned from mice peritoneal macrophages (31, 32). Instead of apoptotic cells, PS coated nuclei ejected from erythroid precursors were used in hemophagocytosis tests. A recent study found TIM4 requirement for β1 integrin interaction for apoptotic cell engulfment (75). A striking property of apoptotic cells is an imposing induction in immune cells such as monocytes–macrophages and certain DC subsets of a tolerogenic–anergic cytokine phenotype including TGF-β1, IL-10, and PGE_2 (42, 67, 71, 76). However, for DC maturation and antigen presentation, a preceding pro-inflammatory phase, possibly to apoptotic antigens, is required. TIM3 ligand galectin-9 was shown to mediate human DC maturation by inducing IL-12 and TNF-α cytokines through NF-κB activation, in synergizing with TLRs (77). By contrast, in Th cells, TIM-3 can have an inhibitory effect on Th1 inflammatory phenotype, as well as on macrophages where it down-regulates IL-12 production. TIM-3 ameliorates murine EAE (78, 79). In inflammatory conditions, such as viral infection, monocyte-derived DCs assume an IL-10 suppressive phenotype through PS dependent hemophagocytosis to relief inflammation (80). Recently, a key Treg subset has been described in mice, expressing C-type lectin receptor CD69 and $FoxP3^+CD69^+$, regulated by STAT5 and ERK pathway (81). CD69 is involved in maintenance of tolerance, in regulation of pro- and anti-inflammatory Th17–Th2 cell phenotypes, and in expression of TGF-β1. Patients with an active SLE were found to be deficient in $CD^{4+}CD25^{high}$ Treg cell suppressive function (82).

Besides the effects on DCs, C1q was previously shown to directly inhibit T cell activation and proliferation via its gC1qR expressed on these cells (54). This receptor is also expressed on B-cells, where it may have tolerogenic effect (83). It has been suggested that this C1q/gC1qR negative regulatory pathway may be involved in maintenance of peripheral self-tolerance to nuclear antigens (46, 63). TIM-3 takes part in apoptotic cell recognition and uptake by DC, in antigen cross-presentation to T cells, in amelioration of autoimmune disease, and in immune cell tolerance induction. TIM-3, together with TIM-4, suppressed autoantibody production (79, 84). TIM-3 negative effects on Th1 appear to be similar to that of C1q/gC1qR showing anergy of T cells (54). TIM-3 is also associated, gC1qR dependently, with regulation of suppressor of cytokine signaling-1 protein (SOCS-1) and of opposing STAT-1 (83, 85).

Clearance by C1q of apoptotic bodies is regarded to be an important protective function, since its impairment is associated with renal, vascular, and other tissue deposition of complement components and of apoptotic bodies. If not rapidly removed, the altered self-debris may become immunogenic, exposing cryptic epitopes, leading to induction of autoantibody production (27, 56, 86, 87). Paradoxically, SLE, despite C1q deficiency, is a complement activation inflammatory disorder. Perhaps, MBL-associated serine proteases (MASPs) coupled with classical pathway may substitute for C1q inflammatory function (43). Alternative pathway may also become involved (86). C1q would thus appear protective against the disease through its putative tolerogenic effects (30, 46). As shown with macrophages, apoptotic cell clearance receptors are separate from the ones mediating tolerance (53). Further supporting this view is the finding in mice deficient in mannose binding lectin (MBL), and in CD14 receptors. Such animals exhibited apoptotic cell and debris deposition in various organs, but did not develop a SLE-like autoimmune pathology (74, 88, 89).

According to self-tolerance hypothesis, C1q and C4 may through immature DCs and immature B cell responses maintain a threshold for negative selection of antigen specific self-reactive B cells (30). In SLE, the main immune targets are highly conserved ribonucleoproteins. Natural IgM producing B cells, specific against these proteins, are kept anergic by C1q, C4, and by Tregs. Inert B cells may escape their apoptosis when unduly activated in the absence of C1q or C4 (29, 30). Using a mouse model of SLE associated with C4 deficiency, it has been revealed that tolerance and anergy of self-reactive B cell clones are impaired, which is normally maintained through interaction with bone marrow myeloid cells, and by spleen negative clonal selection. In the absence of C4, more of such antigen specific B cells reach maturity in peripheral lymphoid compartment, effects furthered by accumulated nuclear debris (90). Perhaps, by appearing so influential in self-tolerance, C1q and C4 might function in epistasis, i.e., being interdependent in their cell signaling.

Self-tolerogenic C1q/gC1qR pathway could potentially endow C1q with a protective function, distinct from that of C1q in apoptotic debris removal (30, 46, 63). These two C1q properties in immature DCs seem to vary in their receptors they may engage, i.e., C1q/gC1qR and C1q/cC1qR, respectively (42, 46, 64). In addition to self-reactive B cell clone elimination, their peripheral suppression by Tregs may be diminished as seen in patients with diseases such as SLE and anti-phospholipid syndrome (APS) (82, 91).

In a physiological steady state, immature DCs of various tissues sample apoptotic cell and other debris in order to migrate to lymphatic tissue where they, as follicular DC, may undergo maturation and immune activation by either presenting specific antigens via MHC class II to naïve T cells, or be tolerized (59). A role of C1q in self-tolerance, as well as in apoptotic cell removal, would be consistent with murine SLE model, rendered deficient in their C1q A-chain gene as homozygotes ($C1q^{-/-}$). Such mice had shortened life span, a quarter developing glomerulonephritis with apoptotic body deposits, and ICs with autoantibodies (92).

C1q, Autoimmunity, and B Cell Tolerance

B cells are major effectors in SLE autoimmunity. Fate of immature B cell stages to maturity is selected via their antigen IgM/IgG membrane receptor (BCR) signaling, and modulated by several positive and opposing co-factors receptor, including T cell help (60, 93, 94). In these events, membrane bound complement

receptor 2 (CR2, CD21), as part of BCR complex, include the signaling co-receptor CD19. CD21 captures C3d/microbe or altered self-antigen complexes, for antigenic-specific BCR stimulation. At the same time, C3d/antigen ligated CD21 activates CD19, which amplifies antigen specific BCR signaling, synergistically with BCR (95). Antigen-sensitivity of BCR complex is further boosted by co-receptor CD21–C3d/Ag complex, which cross-links a number of BCR complexes on a B cell membrane by being polyvalent, e.g., in ratio 3-C3d/1-Ag. Such structures can lower an antigen activation threshold for its BCR, 100- to 10,000-fold (61). C3d/Ag complexes are generated on unprotected body cell or microbe surface antigens, to which C3b-thiol ester domain C3d (TED) makes covalent bonds with Ag-hydroxyl groups. These are captured, processed, and presented in draining lymph nodes to B cells by FDCs. CD19 co-receptor also mediates DC retention in germinal centers and their survival (96). CD19/CD21/BCR complex can be translocated to B cell lipid rafts, which can interact with other signaling receptors (97).

CD19 glycoprotein is involved in intrinsic Src-family kinase signaling, associated with Inositol 1,4,5-trisphosphate 3-kinase (IP3 3-kinase/IP(3)K), Ca^{2+} influx, and mitogen activated protein (MAP) kinase (97, 98). A further BCR co-receptor is Bruton tyrosine kinase (Btk), expressed constitutively, and positively modulating BCR signaling. As tested in CD19 – promoter – Btk transgenic mice, an over expression of Btk within their B cells via NF-κB elicited in such animals a SLE-like anti-dsDNA autoimmunity, showing expansion of specific plasma cells. This phenotype was due to impairment of antigen specific BCR negative selection (99). Btk gene deletion, on the other hand, resulted in programed death 1 (PD1) expression, B cell apoptosis, and in hereditary disease such as X-linked agammaglobulinemia. Normally, Btk may thus directly maintain a threshold on antigen specific B cell negative selection, although its expression modulation itself may be subject to other BCR co-regulators. B cell malignancy such as chronic lymphocytic leukemia (CLL) is associated with a dysregulated Btk, and ameliorated by its inhibitors (99).

CD19-co-receptor is closely associated with CD22, an inhibitory co-receptor of BCR signaling, a member of Siglec family (sialic acid-binding Ig-like lectin). On IgMmBCR ligation and via CD19 Lyn kinase, CD22 is activated through phosphorylation of its immunoreceptor tyrosine based inhibition motifs (ITIMs). These activated motifs then recruit inhibitory tyrosine phosphatases such as SHS-1, SHS-2, and inositol-phosphatase (SHIP), dephosphorylating their substrates, including CD19 cytoplasmic domain tyrosine residues, as a negative feed-back loop (100, 101). CD22 and Siglec-G defect and polymorphism in acetylesterase are associated with susceptibility to autoimmune disease (102, 103).

C1q, which is highly expressed on B cells, may negatively influence BCR signaling, and promote their tolerogenic capacity. It may be part of the negative C1q/gC1qR pathway, which suppresses $CD4^+$ T cell activation and proliferation (54). Earlier studies with B cell lines, such Raji, Daudi, U937, and Molt4, demonstrated a C1q-induced anti-proliferative response (55). C1q promoted human peripheral blood and tonsillar B cell differentiation, stimulated with *S. aureus* Cowan (SAC), but did not affect their proliferation. C1q specifically induced IgG production over that of IgM, via its collagen domain, bound to B cell surface (20). Apparently both C1q receptors may be engaged in B cells with separate functions, depending on B cell stage and stimulation (20).

In a comparative study on C1q and on HCV core protein effects, via gC1qR ligation, C1q likewise did not enhance normal human $CD20^+$ B cell proliferation, stimulated by phytohaemagglutinin (PHA). However, C1q, like HCV core protein, augmented CD69 expression, which is an activation marker and an immune suppressor (81, 83). By contrast, HCV core protein augmented B cell proliferation, co-stimulatory molecule expression, down-regulated suppressor of cytokine signaling-1 (SOCS-1), and up-regulated STAT-1 activation-phosphorylation. In T cell, it had opposite effects. These responses were gC1qR dependent, and associated with TIM-3 function. Such cell signaling dysregulation is thought to sustain the viral disease chronicity and autoimmunity (83, 85). C1q/gC1qR effects on T and B cells may be compatible with C1q being a tolerogenic protein against autoimmunity as in SLE (63). Its stimulatory effect on B cell CD69 expression may be relevant. CD69 is a type 2 lectin receptor, expressed on all bone marrow derived leukocytes. Its co-expression on a Foxp3 Treg subset facilitates its tolerogenic property. Potentially, it may also augment such effects of CD11b/CD22 receptors in B cell tolerance (104). CD69 is a negative regulator of leukocyte migration-egress of lymphocytes from lymphoid organs, inhibiting sphingosine 1-phosphate receptor-1 ($S1P_1$) (81, 105). gC1qR is a multi-ligand binding adaptor protein to various cell receptors (45). In adipocytes, it serves as a positive co-receptor to insulin receptor tyrosine kinase in insulin signaling (106). In B cells, it might be a tolerogenic co-receptor.

Autoantibodies Against C1q

Autoantibodies against C1q collagen region have been strongly correlated with lupus nephritis, suggesting their pathogenicity (107). In a mouse model, it was revealed that anti-C1q autoantibodies were glomeruli damaging only in conjunction with complement fixing ICs with anti-nuclear protein and dsDNA antibodies deposits (108). Apparently, C1q also exhibits hidden epitopes when bound to various cell debris (107, 109). Anti-gC1q autoantibodies were detected in several SLE nephritis patients, which inhibited C1q binding to IgG and C-reactive protein. It has been suggested that such autoantibodies may represent an acquired C1q deficiency (110, 111). Secondary C1q deficiency is much more common in SLE and other diseases with variable frequency. However, only 20–50% of SLE patients have anti-C1q autoantibodies. Some disease free people also have anti-C1q autoantibodies, suggesting a need for an injury as a trigger. To sustain the disease, however, anti-nuclear antibodies and ICs, also mediated largely by C1q, are a hallmark for SLE (107).

Thus, C1q may distinctly contribute to apoptotic cell phagocytic clearance as well as tolerance maintenance to such natural self-antigens, effects which may be cooperative. C1q may employ in these tasks its cC1qR as well as its gC1qR in negative anti-inflammatory pathway of T and B cells. Both C1q receptor expression is required in DC differentiation and consequently in antigen presentation to T and B cells. On debris clearance, DCs may assume a tolerogenic, anergic state. Antigen stimulated T cells and B cells may respond similarly to C1q negative regulation.

Autoimmune disease, such as SLE, is heterogeneous and complex. Its susceptibility may be increased not only by C1q defects but also by malfunction of other genes such as of BCR co-receptor Btk, CD22, TIM-3, and Tregs in BCR signaling. There is clearly a gene redundancy in apoptotic cell clearance mechanism. Thus, there is a good argument to explore the negative C1q/gC1qR pathway further in autoimmune disease.

Role of C1q in Pregnancy

Activation and Regulation of Complement in Human Pregnancy

Healthy normal pregnancy is associated with systemic activation of complement system (112–114). Placenta with the semi-allogenic fetal tissue is a potential target for complement-mediated immune attack (115, 116). During normal pregnancy, complement system is found activated by the presence of natural IgG in the normal placenta. This needs to be well regulated to control alloreactivity and placental inflammation (117). Complement products are found deposited on the placental tissues (118–121). Following decidual invasion of the fetal tissues, the fetus gets exposed to the complement system in the maternal blood at the intervillous space (122). In addition, extravillous trophoblasts (EVT), known as endovascular trophoblasts, migrate down the luminal walls of the spiral arteries and eventually enter the decidua and cause deep vascular changes in the spiral arteries producing apoptotic debri that favors complement activation with limited placental damage related to vascular remodeling process (123, 124). However, excessive or uncontrolled activation of the complement system can provoke adverse outcomes damaging the fetal tissues. Thus, for successful pregnancy, complement activation requires proper regulation.

During normal pregnancy, fetus specific antibodies such as anti-HLA and anti-trophoblast antibodies in maternal serum bind C1q and activation of the classical pathway can have adverse pregnancy outcomes (125, 126). A number of complement regulators including factor H (117), decay-accelerating factor (DAF, CD55) (127), membrane co-factor protein (MCP, CD46), and CD59 (128, 129) are expressed locally on the surface of the cytotrophoblasts, syncytiotrophoblasts coating the villi, and EVT (130, 131). Thus, complement system at the feto-maternal interface defends the mother and the fetus against the invading pathogens; protects the fetus from the maternal immune system and maintains fetal tolerance (**Figure 2**).

Distribution of C1q in the Female Reproductive Tract

The levels of complement components gradually rise with gestation. C3, C4, C5, factor B, factor H, and factor I have been detected in amniotic fluid, umbilical cord blood (132–134), utero-placental spiral arteries (135), and placental tissue (136). Recently, deposition of early (C1q and C4) and late (C5, C6, C9) complement components has been reported in the placenta (123). Most notably, early components are detected in the stroma of the villi around fetal vessels, while late complement components are detected on the trophoblast membranes (118). Spiral arteries in the first trimester decidua show both early and late complement component deposits. C1q is produced by the trophoblasts and decidual endothelial cells (DECs) (118), human decidual stroma, and synthesized invasive EVT (118, 137). Recently, non-immune decidual cells such as invasive trophoblasts and stromal cells have been shown as a major source of C1q at the feto-maternal interface using first trimester human placenta (138).

Role of C1q at the Fetal–Maternal Interface During Pregnancy

C1q is considered to have a key role in trophoblast invasion, spiral artery remodeling, and normal placentation (139). C1q is produced by DECs that connect the endovascular trophoblasts and the endothelial cells required for normal placentation such as trophoblast invasion into the decidua and vascular remodeling during pregnancy (118, 137, 140). In addition, it enhances trophoblast adhesion and migration by the activation of MAP kinase pathway via gC1qR/P33 integrin (137). Recently, transcriptional factor, PU.1 has been shown to be associated with the decidual C1q expression in trophoblasts and stromal cells. PU.1 transcriptional factor is associated with the development of hematopoietic myeloid lineage immune cells (141). Thus, the presence of PU.1 and C1q at the feto-maternal interface is likely to be involved in the regulation of trophoblast and stromal cell linage differentiation during early stages of pregnancy such as implantation and placentation (142).

Association of C1q with Disorders and Complications of Pregnancy

Excessive complement activation has been associated with adverse pregnancy outcomes such as miscarriages, preterm delivery, and preeclampsia. During pregnancy, EVT invades the decidua until it reaches the inner third of the myometrium. The extent of trophoblast invasion needs to be regulated. Inadequate tapering of maternal arteries and improper trophoblast invasion has been associated with preclampsia. It is a common disorder characterized by abnormal placentation. $C1q^{-/-}$ mice show key features of preeclampsia, such as hypertension, albuminuria, endotheliosis, decreased placental vascular endothelial growth factor (VEGF), increased soluble VEGF receptor 1 (sFlt-1), increased oxidative stress, decreased blood flow, increased fetal death, diminished litter size, abnormal invasion of trophoblasts, and increased levels of STAT-8 (inhibitor of trophoblast migration) (139). Furthermore, $C1q^{-/-}$ mice are unable to clear apoptotic trophoblasts whose accumulation can result in abnormal placentation (92). Differences in the C1q deposition in placental samples between early and late-onset of preeclampsia groups can act as a good biomarker for preeclampsia (143). It appears that C1q produced by trophoblasts are responsible for impaired trophoblast migration and abnormal placentation. It is also likely that the trophoblast C1q is required for the cross-talk between the EVT and DEC to promote trophoblast migration, vascular remodeling, and normal placentation process (140) (**Figure 3**).

Normal pregnancies are characterized by increased anti-C1q autoantibodies in the serum when compared to complicated pregnancies. Ectopic pregnancies have the lowest levels of anti-C1q

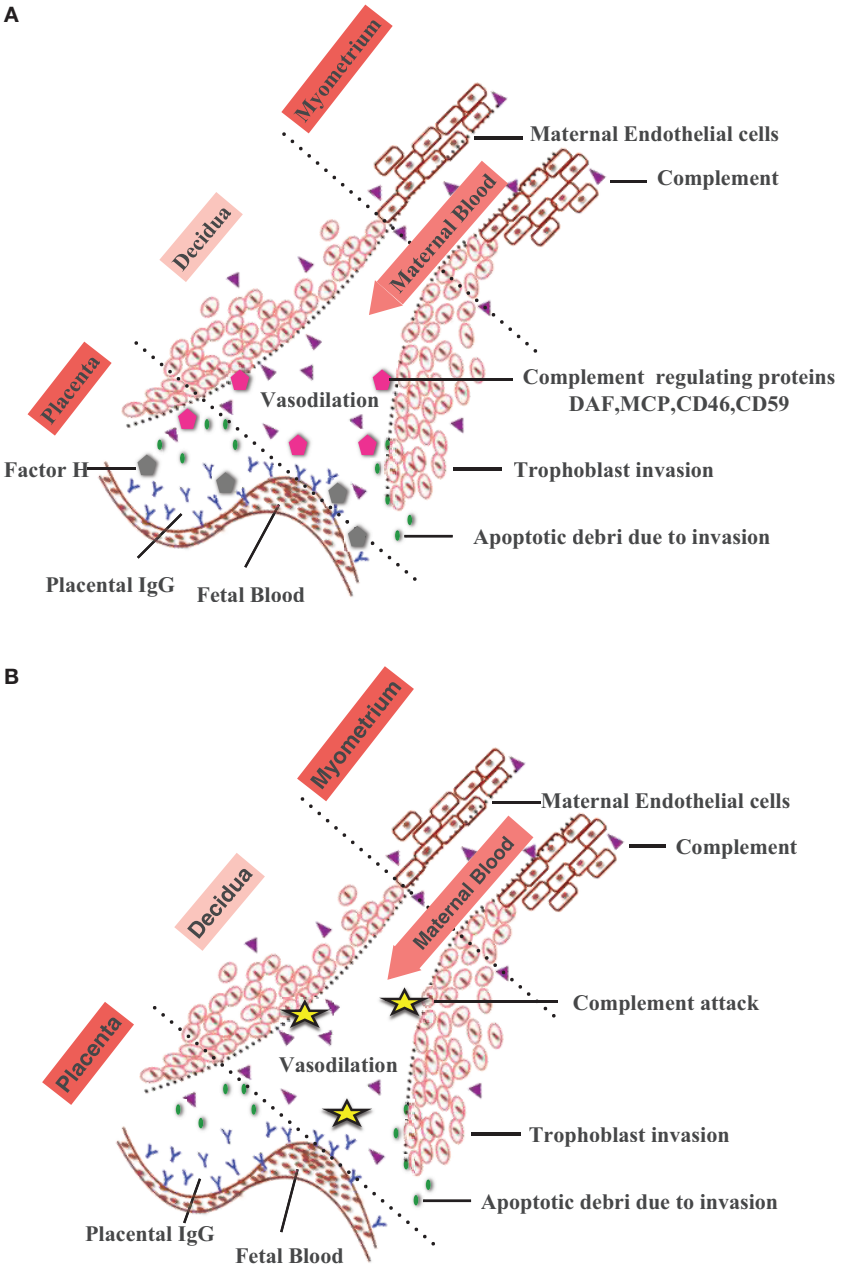

FIGURE 2 | Complement activation at the feto-maternal interface during pregnancy. **(A)** During normal pregnancy, maternal endometrium transforms into feto-maternal interface (decidua) and surrounds the implanted embryo offering nutrition and protection against the maternal immune system. With advancing gestational age, decidua undergoes extensive tissue remodeling (trophoblast invasion and spiral artery formation) that results in apoptotic debris formation. The apoptotic debris and placental IgG activate classical pathway via C1q deposited at the feto-maternal interface. By eliminating the immune complexes and apoptotic cells, complement system protects the mother and fetus. Complement regulatory proteins found on human placental tissues such as Factor H, DAF, MCP, CD46, and CD59 prevent excessive complement activation. **(B)** In the absence of regulatory proteins (DAF, MCP, CD46, CD59), excessive complement activation results in improper placentation, characterized by increased ROS, inflammatory cytokines, angiogenic proteins (accumulation of apoptotic cells), improper vascularization, and spiral artery remodeling contributing to development of pathological/complicated pregnancies.

antibodies with increased IL-15. Thus, IL-15 and anti-C1q antibodies are good serum biomarkers for missed abortions (144). Clinically, spontaneous miscarriage, still birth, and fetal resorption have been detected in mothers exposed to polychlorinated biphenyls (PCBs) (145). In patients with spontaneous abortion, PCBs content is associated with increased expression of gC1qR, and reduced percentage of apoptotic cells. Thus, gC1qR may be relevant for PCBs-mediated trophoblast cell apoptosis (146). Villitis of unknown etiology (VUE), characterized by inflammation in the chorionic villi, has been associated with miscarriage (147).

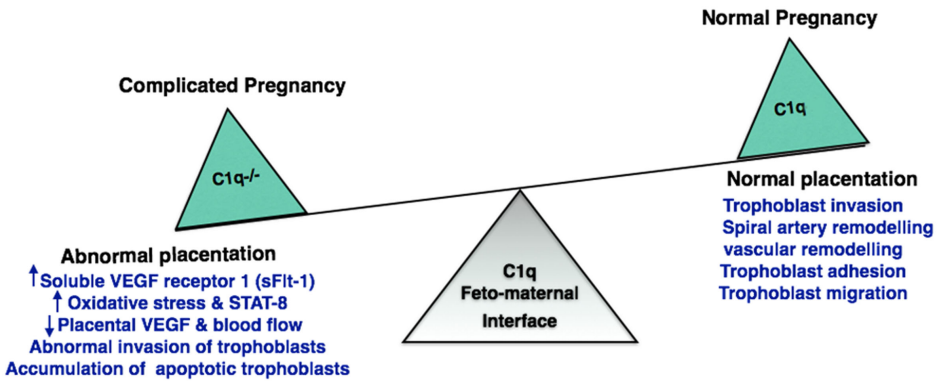

FIGURE 3 | Significance of C1q in normal and adverse pregnancy. Absence of C1q results in abnormal invasion of fetal trophoblast into the decidua. C1q deficiency increases oxidative stress and accumulation of apoptotic trophoblasts. This has an adverse effect on placenta inhibiting the generation of placental vascular endothelial growth factor (VEGF) and blood flow, resulting in implantation failure and pregnancy complications such as recurrent pregnancy loss, miscarriage, abortion, and preeclampsia.

In a study using placental samples with and without VUE, the distribution and amount of C1q, C3d, IgG, and IgM in the chorionic villi were examined. The only difference observed between two groups was the abnormal distribution of C1q in the stromal villi (148), suggesting involvement of C1q in villitis.

Several pathogens including *Trypanosoma cruzi*, cytomegalovirus, *Listeria monocytogenes*, and *Neisseria gonorrhea* can infect the decidua, and then spread to placenta and chorioamniotic membrane. When left untreated, the chorioamnionitis can lead to preterm birth, intrauterine growth retardation, still birth, and other complications of pregnancy (149). *T. cruzi* protozoan infection that spreads to humans by blood sucking bug *Triatominae* (150) can be transplacental, being passed from the mother to the fetus (151). When the mother is infected, the *T. cruzi* calreticulin (TcCRT; 45 kDa), migrates from the endoplasmic reticulum to the external surface of the parasite and binds to the maternal C1q (152). TcCRT bound maternal C1q recognizes/binds to CRT in the placenta. Thus, TcCRT bound maternal C1q acts as a molecular bridge to spread *T. cruzi* infection from the mother to the placenta. TcCRT, a potent virulence factor, enhances infectivity in the host by preventing the activation of the classical pathway and angiogenesis (153, 154). By enhancing the cross-talk between the CRT found on the surface of the parasite and its receptor on human placenta, maternal C1q is thought to play crucial role in spreading *T. cruzi* infection (155). During pregnancy, there is a possibility of increased disseminated gonococcal infection. Fetus remains at an increased risk due to neonatal sepsis, arthritis and systemic illness. Under *in vitro* conditions, C1q incubated with *N. gonorrhoeae* increases the gonococcal cell infection instead of clearing the gonococcal bacteremia (156). DECs have previously been shown to synthesize C1q during pregnancy (118). DECs, as opposed to endothelial cells isolated from adult skin, display decreased levels of TLR-4, MD2, and MyD88. This raises the possibility that C1q at the feto-maternal interface may contribute to immunoregulation of excessive inflammation caused by infection during pregnancy (157).

Potential Therapeutic Agents to Prevent C1q-Associated Pregnancy Disorders

Heparin, a well-known anti-coagulant, has been shown to reduce pregnancy complications in women with anti-phospholipid (aPLs) antibodies (158, 159). It prevents apoptosis of human trophoblasts (160), enhances placental proliferation (161), and reduces EVT invasion (162). Heparin binds to the C1q collagen region following gC1q binding to ICs, thereby reducing the classical pathway activation (163, 164).

Animal studies have shown use of statins as a promising therapeutic strategy in preventing the onset of preeclampsia. Pravastatin treatment in $C1q^{-/-}$ mice prevents the key features of preeclampsia by lowering blood pressure, and improving vascular reactivity, endothelial function, and normal placental VEGF. The study highlights the effectiveness of provastins in order to improve placentation and pregnancy outcomes (139).

Role of C1q in the Pathophysiology of the Central Nervous System

Many components of immune system including complement proteins are locally produced in the CNS in order to maintain homeostasis. Similar to its role in the peripheral system, C1q is associated with protection of the CNS against infections such as bacterial meningitis. Low-level complement activation is also considered to be beneficial in the removal of toxic or aggregated proteins. However, sustained or chronic activation can be detrimental to the CNS due to microglial activation and production of pro-inflammatory cytokines. Therefore, neuroinflammation plays a role in the maintenance of homeostasis or neuroprotection as well as neurotoxicity, depending upon the level of complement activation (165–167). Apart from having an important role in CNS infections, traumatic brain injury, neurodegenerative diseases, and other neurological and psychiatric conditions (**Table 2**; **Figure 4**), a novel role for C1q has been established in the pathogenesis of prion diseases, development of CNS and synaptic pruning.

TABLE 2 | Role of C1q in CNS diseases.

CNS disease	Role of C1q	Reference
Prion diseases	Initial uptake of PrP from gut; propagation and transmission to CNS	(166)
Alzheimer's disease	C1q binds to β-amyloid via B chain of gC1q region and modulates phagocytosis by microglia	(2, 168)
	Absence of C1q causes less neuropathology in AD mouse models	(169)
	Co-localization of C1q with neurons is observed in pre-clinical stage of AD	(170)
	Increase in reactive oxygen species in neurons incubated with C1q leading to neurotoxicity	(171)
	In the presence of C1q, the neurotoxic effects of Aβ and SAP are reduced	(48)
Parkinson's disease	Neuromelanin opsonized by C1q and phagocytosed by C1q-positive microglia	(172)
Multiple sclerosis	Co-localization of C1q with reactive astrocytes in CNS plaques	(173)
Schizophrenia	Increase in serum C1q and increased total complement activity in serum	(174)
Spinal cord injury (SCI)	Deficiency of C1q improves recovery following SCI	(175)
Stroke	Presence of C1q in ischemic lesions	(176)
	C1q deficiency found to be neuroprotective in hypoxic–ischemic brain injury	(177)
Huntington's disease	Increased C1q co-localization in striatum	(178)

Microglia, astrocytes, and neurons are known to be good sources of complement proteins especially under stress, injury, ischemia, or infection. Microglia are the specialized macrophages in the CNS that constantly survey their surroundings. Upon being activated by environmental or external stimuli, microglia retract their ramifications and appear amoeboid. Production of C1q by microglia is at low level in the resting phase, which increases upon activation, leading to production of pro-inflammatory cytokines that can cause neuronal cell death (179). C1q binds to apoptotic cells and neuronal blebs thereby enhancing phagocytosis by microglia. Farber et al. (180) found that C1q stimulated microglial synthesis of TNF-α and IL-6, which is considered to disrupt blood–brain barrier. Moreover, C1q released by activated microglia maintained and balanced the level of activation of microglia in an auto- or paracrine manner. Interestingly, microglia stimulated with C1q *in vitro* were found to decrease LPS-induced production of TNF-α and IL-6 (181). Astrocytes have been shown to produce C1q in response to cerebral fungal infection (182). Astrogliosis refers to change in morphology and hypertrophy of astrocytes, which leads to scar formation. This occurs in response to CNS insult and is associated with C1q and cytokine secretion. Furthermore, co-localization of C1q with reactive astrocytes is a significant feature of CNS plaques seen in multiple sclerosis patients (173). Neurons also produce C1q *in vitro* (183). For example, hippocampal cells secrete C1q in response to β-amyloid (184). Presence of C1q protects neurons against β-amyloid and serum amyloid P-induced neurotoxicity (48). C1q-mediated neuroprotection involves up-regulation of genes associated with cholesterol and lipid distribution/metabolism such as cholesterol-25-hydroxylase and insulin induced gene 2. Addition of C1q leads to decreased levels of cholesterol in neurons, which is known to be a factor that causes outgrowth of neurites. C1q also directly affects nerve growth factor (NGF) by upregulating its transcription factors and downregulation of microRNA that target NGF (185). Thus, C1q (and the complement system) has a dual role: beneficial as well as detrimental to the CNS offering opportunities for therapeutic intervention in neuroinflammatory and neurodegenerative diseases.

Prion Diseases

Prion diseases or transmissible spongiform encephalopathies are a group of neurodegenerative diseases that are infective and are known to occur in humans, cattle, deer, and other livestock such as sheep and goat. Some of the diseases that affect humans include Creutzfeldt–Jakob disease (CJD), fatal familial insomnia, and kuru; while those involving animals include bovine spongiform encephalopathy and scrapie. Prion proteins are normally present in human body, although the fibrillar form of this prion protein (PrP^{Sc}: scrapie-associated prion protein) are cytotoxic and form protease resistant fibrils and oligomers. PrP^{Sc} accumulates in neurodegenerative disease plaques. Histopathological features observed in prion disease affected brains include spongiform changes, gliosis, loss of neurons, accumulation of amyloid plaques, and synaptic degeneration in hippocampus (186–188).

C1q is involved in uptake, propagation, and transmission of PrP^{Sc} from the gut to the brain. With the exception of direct brain exposure due to neurosurgery, the mode of transmission for prions is through oral or parenteral route. $C1q^{-/-}$ or $C3^{-/-}$ mice have been found to have significantly delayed onset of disease after peripheral exposure to PrP^{Sc} (49). C1q plays a role in the initial uptake of PrP^{Sc} by conventional DC that express C1q receptor calreticulin (50). Absence of C1q has been found to significantly reduce the capture of prions by DC (189). Additionally, follicular DC is also considered to be a good source of endogenous monomeric prion protein. C1q interacts with PrP^{Sc} in an antibody-independent manner, which is considered to proliferate on the surface of follicular DC (190). Inactivation of follicular DC was found to delay neuroinvasion by PrP^{Sc} (191). *In vitro* studies using human microglial cells have also shown that the presence of C1q along with PrP causes an increase in fibril formation of PrP. This leads to an increased activation of microglial cells causing secretion of pro-inflammatory IL-6 and TNF-α (192). C1q has been found to enhance formation of PrP oligomers and form complexes with these PrP oligomers via its gC1q domain. This complex has the ability to activate classical pathway, and this is considered to prevent neuronal cell death caused by these oligomers of PrP (51). However, this interaction is considered to prevent the elimination of prion seed, thereby increasing its aggregation. Thus, C1q is involved in initial pathogenesis of prion disease by enhancing uptake of prions in the peripheral system, propagating into CNS, forming complexes with PrP oligomers, and increasing fibril formation.

Synaptic Pruning

C1q (and the complement system) has been shown to play a vital role in synapse elimination during developmental stage of CNS

FIGURE 4 | Various diseases of central nervous system and the involvement of C1q. various functions of C1q in the central nervous system and the associated diseases are illustrated. Increased levels of C1q have been found in Huntington disease and schizophrenia. In Huntington disease, striatal volume is decreased in mouse models; however, cell number does not differ. Prion disease is a result of undue loss of neurons and misfolded prion protein, PrP^{Sc}, deposition. Early synaptic failure and neuronal loss affect behavioral symptoms, indicating the early onset of disease. C1q deficiency reduces the capture of prions by DCs, as it is only involved in the primary phase of the disease, transmitting prions to the CNS. C1q production is triggered in the CNS by astrocytes in response to cerebral fungal infection. The role C1q plays in Parkinson's disease includes the opsonization of neuromelanin and phagocytosis by microglia cells. Complement pathway up-regulation in brain regions can be observed in AD, which may be due to amyloid β peptides binding within collagen-like domain of C1q, thus activating classical pathway. In addition, fibrillar amyloid β interaction with C1q via activates the classical pathway. Microglia cells, which express C1q in the substantia nigra pars compacta of Parkinson disease, phagocytose and clear debris of degenerating neurons.

as well as in synaptic pruning to increase efficiency of neuronal transmission. During developmental stage, a redundant growth of neural network occurs which also leads to excessive synaptic formations. This needs to be kept in check in order to maintain plasticity and ensure proper functioning of the CNS. Complement proteins, including C1q, have been localized at the sites of synaptic

elimination in the mouse reticulogeniculate system, which is a model for studying synapse elimination during developmental stage (47). C1q is involved in synapse elimination during the developmental stage of CNS where postnatal neurons express C1q in response to an astrocyte-mediated signal and then co-localizes with developing synapses. $C1q^{-/-}$ mice show failure of synapse elimination (47). Thus, role of C1q is considered to be akin to that in the peripheral system wherein C1q tags apoptotic cells. In the CNS, C1q is considered to tag immature or unwanted synapses leading to their elimination by microglia.

An important element of glaucoma includes death of retinal ganglionic cells (RGCs). In mouse retina, C1q is expressed only during the developmental phase. Interestingly, in glaucoma mouse model, C1q re-localizes to synapses in adult inner plexiform layer of the retina. This mechanism could be an important early event in glaucoma leading to C1q-mediated tagging of synapses, which, in turn, causes synapse loss and death of RGC. Furthermore, *C1qA* and *C1qB* chain gene expression was found to be markedly elevated (up to 25-fold) in mouse retinas with moderate glaucoma (47). More recently, TGF-β, secreted by astrocytes, has been shown to induce production of C1q by purified RGC (193). Disruption in cytokine signaling pathway of TGF-β leads to inhibition of synaptic pruning *in vivo* that is dependent on C1q and microglia. Furthermore, C1q has been also found to regulate microglia-mediated synaptic pruning in the thalamus (193).

Failure of synaptic pruning is an essential aspect of epileptogenesis. $C1q^{-/-}$ mice show aberrant synaptic connectivity and spontaneous epileptiform activity is evident (194). This is likely due to the presence of excessive excitatory synapses. This failure of synaptic pruning also contributes to a significant increase in dendritic length, branching, and density of dendritic spines in $C1q^{-/-}$ mice (195). In a rat model of temporal lobe epilepsy, an increase in C1q production by glial cell and neurons was observed in hippocampus following status epilepticus (196). Role of C1q in epilepsy is currently ambiguous. Due to an increased activation of complement, neuroinflammation can reduce threshold for seizures. On the flipside, its absence leads to spontaneous epileptiform activity due to deficient synaptic C1q function.

An increase in C1q level (up to 300-fold) has been observed in aging brain, especially in pyriform cortex, substantia nigra, and hippocampus (197). C1q immunoreactivity was detected in microglia throughout the early postnatal brain. Interestingly, C1q also co-localized with inhibitory (GABAergic) neurons in the hippocampal dentate gyrus. C1q was also abundant in close vicinity of synapses. Furthermore, the increase in C1q level in aging brain also correlated with cognitive decline. Indeed, synaptopathy or synaptic dysfunction is considered to be the one of the earliest feature of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and Prion diseases (198, 199).

Induction of Apoptosis in Cancer Cells by C1q

Human prostate cancer cells DU145 express WOX-1, which is known to be a tumor suppressor and pro-apoptotic protein. When DU145 cells cultured overnight in the presence of heat-inactivated serum (10%) followed by starvation for 1 h without serum were treated with exogenous C1q, it induced rapid accumulation of nucleic Tyr33-phosphorylated WOX-1 (p-WOX1) in comparison with the C1q-depleted serum treated cells where p-WOX1 was mainly localized in the cytoplasm (38). The C1q-treated DU145 cells adhered to the cover glass surface and a significant increase in the formation of clustered microvilli in between the cells was observed. Subsequently, the cell membrane shrunk, membrane blebbed, and eventually the cells underwent apoptosis. The internucleosomal DNA fragmentation analysis showed the cleaved DNA ladders, which further established the C1q/WOX1-induced apoptosis in the DU145 cells.

The activation of WOX1 by C1q was further determined by culturing the EGFP–WOX1 and EGFP alone transfected DU145 cells overnight (in 10% heat-inactivated fetal bovine serum) followed by C1q treatment for 24 h. The C1q treatment resulted in activation of WOX1-induced apoptosis and growth suppression of the DU145 cells in the EGFP–WOX1 transfected cells whereas C1q did not induce apoptosis in the DU145 cells overexpressing EGFP vector only. These findings also suggest that WOX1 was a downstream effector of C1q-mediated apoptosis without the involvement of complement activation since the serum was heat inactivated.

Out of the two domains, N-terminal WW and C-terminal short chain alcohol dehydrogenase/reductase (SDR) of WOX1, the WW domain is believed to be involved in the C1q-induced activation of WOX1. This was shown by transfecting the DU145 cells with a dominant negative-WOX (dn-WOX1), which was designed with alterations in the N-terminal WW and is known to block the apoptotic function of p53 and prevent phosphorylation of endogenous WOX1 at Tyr33. The transfected cells resisted C1q-induced apoptosis when transiently overexpressed with dn-WOX1 (EGFP tag) whereas non-transfected cells underwent apoptosis. Furthermore, co-transfection of DU145 cells with N-terminal WW domain of WOX1 (WOX1ww) and dn-WOX1 showed a decrease in the C1q-induced apoptosis. These observations suggest that N-terminal WW domain of WOX1 and the Tyr33 phosphorylation in WOX1 play an important role in C1q-induced apoptosis.

To determine the role of p53 and WOX1 in C1q-regulated cell death, DU154 cells were transiently overexpressed with both p53 and WOX1. The DNA fragmentation significantly increased in the combination compared to p53 or WOX-1 alone expressing cells, suggesting that tumor suppressor p53 physically interacts with WOX1 to induce apoptosis. However, alterations of human *WOX* gene occur most frequently in the prostate. Therefore, prostate tissues were examined for the expression of C1q. The immunofluorescence comparison of the age-matched prostate tissue with the benign prostatic hyperplasia (BPH) and prostate cancer showed that C1q was significantly down-regulated in the later tissues. These findings raised a question whether down regulation of C1q *in vivo* may reduce the activation of tumor suppressors, which would subsequently result in better survival of the prostate cancer cells. Therefore, when DU145 cells were cultured overnight under serum free conditions, in the presence of 1% normal human serum

or 1% human serum deficient in C1q, C6, C7, C8, or C9, it was noted that C1q and C6 were essential for the expression of WOX2 (isoform of WOX1) and p-ERK and remainder conditions did not support the expression of these proteins. These observations were further confirmed by immunofluorescence microscopy and western blot that showed the downregulation of p-WOX1 under C1q or C6 free conditions, and hence no apoptosis was seen. Similarly, p53 nuclear accumulation was reduced in the C1q or C6 free conditions whereas C9 free conditions promoted the accumulation of p53 in the nuclei suggesting that C9 restricts the p53 activation. C1q and C6 were also shown to block JNK1 activation, which is known to block the apoptotic function of WOX1 *in vitro*.

In addition, hyaluronic acid was shown to activate STAT3 in the DU145 cells when cultured in the C1q deficient conditions, which causes metastasis by up regulating the STAT3 phosphorylation, suppression of p53, and WOX1 activation. The induction of apoptosis by C1q in the cells overexpressing WOX1 such as breast cancer cells (MCF7), neuroblastoma SH-SY5Y, and SK-N-SH cells was also established. It appears that C1q plays a key role in the activation of tumor suppressor WOX1, which is required for blocking the cancer cell proliferation. C1q did not enhance the p53-mediated apoptosis; however, p53 and WOX1 balanced binding interaction results in their synergistic effect in inducing apoptosis in response to the C1q treatment. The downregulation of C1q enhances prostate hyperplasia due to a failure of WOX1 activation failure and increased activation of STAT3. It is worth pointing out here that the pro-apoptotic effect of C1q observed on human prostate cancer cells is an exceptional and novel phenomenon reported. C1q has often been detected at tumor site where its expression is associated with tumor progression.

Role of C1q in Aging

Serum C1q concentration and expression increases with aging, which causes impaired regeneration capacity of various tissues by activating canonical Wnt signaling pathway (33). Wnt signaling is known to play a key role in tissue development including stem cell self-renewal, degenerative diseases, and carcinogenesis. During canonical Wnt signaling pathway, Wnt binds to the two types of receptors, Frizzled (Fz) family of serpentine proteins and the single-transmembrane protein low-density lipoprotein receptor-related protein 5/6 (LRP5/6), which causes the disruption of the β-catenin destruction complex by recruiting the Axin component of destruction complex to the cytoplasmic tail of the Wnt co-receptor LRP. The β-catenin destruction complex includes proteins: Axin, adenomatosis poluposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3), and casein kinase 1 α (CK1α). Therefore, canonical Wnt signaling pathway causes the β-catenin cytosolic accumulation and translocation into the nucleus to act as a transcriptional co-activator of the T cell factor/lymphoid enhancer factor (TCF/LEF) family (**Figure 5**).

C1q has been shown to activate the canonical Wnt signaling by binding to the Fz receptor and stimulating C1s-dependent cleavage of the ectodomain of LRP6. This C1q-induced activation of Wnt signaling subsequently stabilized the cytosolic β-catenin and increased the expression of Axin 2, a known target gene of canonical Wnt signaling. A heterologous competition assay demonstrated that C1q competed with Wnt for binding to Frizzled-8 (Fz8) cysteine-rich domain (CRD) with comparable affinity, however, 200-fold higher EC_{50} of C1q was observed than Wnt3A. This observation was further supported when an increase in the levels of *Axin 2* mRNA was observed in various tissues of the 2 years old wild-type mice compared with 2 months old and $C1q^{-/-}$ mice, suggesting the relevance of C1q-induced canonical Wnt signaling activation in the aged animals. Similar trend was observed in the isolated satellite cells and fibroblasts from the muscles of young and old mice treated with C1q suggesting a decreased regenerative capacity of skeletal muscles. In addition, with increasing age, an increased amount of the cleaved product of LRP6 was detected in the serum of wild-type mice but not in $C1q^{-/-}$ mice. These observations strongly suggest the physiological relevance of C1q-induced activation of Wnt signaling. However, the extent of this activation is dependent on various factors such as concentration of C1q, target cell exposure, expression level of Fz receptors, LRP5/6 co-receptors, C1r, C1s, and C1 inhibitor in the target cells. In conclusion, the serum C1q concentration increases with aging, which induces canonical Wnt signaling pathway and thereby C1q mediates impaired regenerative capacity of skeletal muscle in aged animals.

Role of C1q in Wound Healing

C1q deposited on the endothelial cells of wound healing skin play a crucial role in promoting angiogenesis by inducing permeability, increased rate of proliferation and tube formation (39, 118). The presence of C1q in the endothelium and stroma of the granulated tissue and its absence in the intact skin was confirmed by immunmohistochemical analysis. C1q transcripts could be seen in stromal cells and vascular endothelium of the lesions, whereas it was undetectable in the cells of intact skin. C1q (10 μg/ml) has been shown to promote permeability, proliferation, and migration of endothelial cells. This effect of C1q appears to be mediated via its gC1q domain.

When C1q was tested using *in vitro* tube formation assay that involves growing endothelial cells and staining them for actin to visualize tube formation, C1q, like VEGF, induced marked changes in the cell pattern, with the formation of tubules assembled by elongation and joining of endothelial cells (200, 201). An *ex vivo* model of rat aortic ring assay was used to analyze the effect of C1q on vessel sprouting from aortic rings. The microvessels formation induced by C1q was visible after 6 days (more apparent after 9 days).

In a mouse model of wound healing using $C1q^{-/-}$ mice, the animals were sacrificed after 14 days post-surgery and skin samples were collected to analyze for the presence of blood vessels. A limited number of small vessels were observed in the wound healing of the $C1q^{-/-}$ mice in comparison to the normal angiogenesis observed in wild-type control. Local application of C1q to the wound of $C1q^{-/-}$ restored vessel formation to the level found

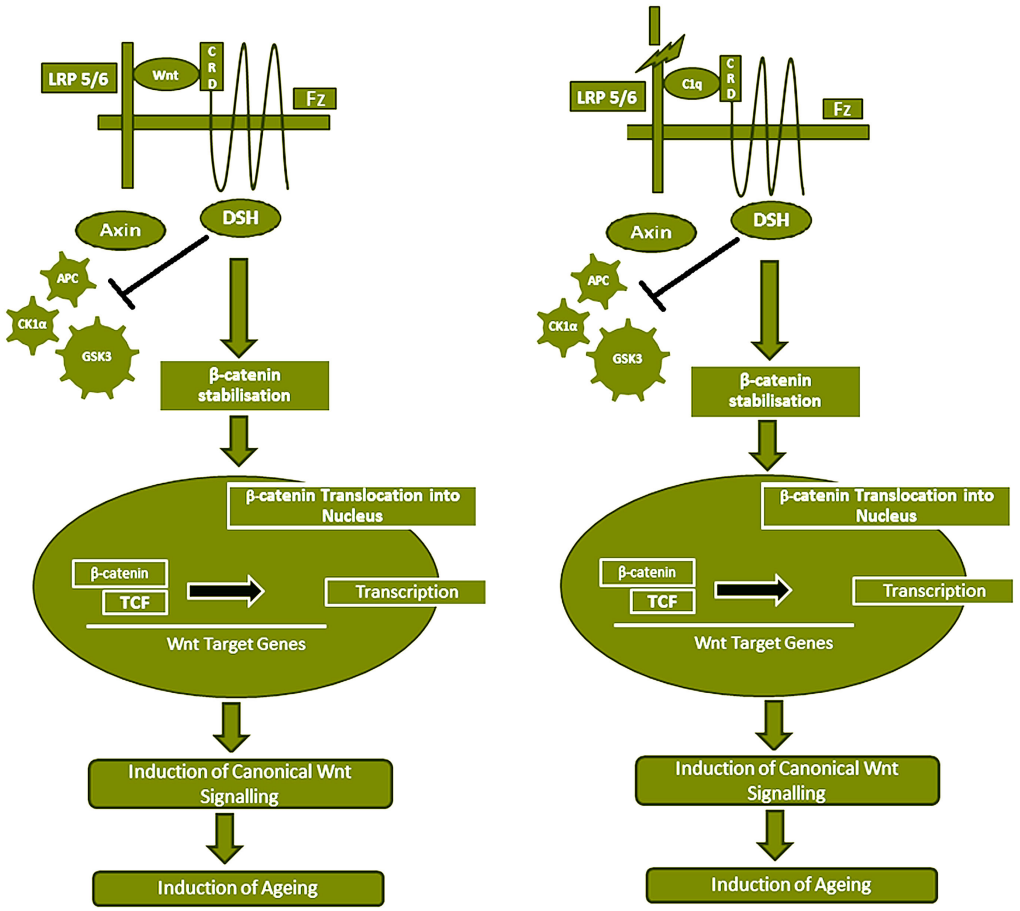

FIGURE 5 | Serum C1 competes with Wnt for Fz receptor binding, resulting in Wnt canonical pathway activation. Wnt binds to Fz receptor and LRP5/6 which causes β-catenin destruction complex (APC, PP2A, GSK3, and CK1α) by recruiting the Axin component of destruction complex to the cytoplasmic tail of the Wnt co-receptor LRP. This results in stabilization and accumulation of β-catenin in the cytoplasm, which eventually gets translocated into the nucleus to act as a transcriptional co-activator of the TCF/LEF family and the Wnt canonical pathway is activated, which has been shown to induce aging. C1q concentration in the serum increases with age. C1q competes with 200-fold higher binding affinity with Wnt for Fz receptor and stimulates C1s-dependent cleavage of the ectodomain of LRP6. The C1q binding to Fz receptor also blocks the β-catenin destruction complex (APC, PP2A, GSK3, and CK1α) and results in stabilization of β-catenin in the cytoplasm, which is then translocated into the nucleus. Subsequently, canonical Wnt signaling is activated.

in WT mice (39). It appears that this angiogenic property of C1q is complement-independent.

Perspectives

C1q plays an important role in the clearance of pathogens. C1q also binds to Gram-positive (*S. pyogenes*) and Gram-negative (*E. coli*) (**Table 1**) and facilitates clearance of bacterial cells (34). However, it can also be used as an escape mechanism by pneumococci, which enhances adherence and invasion in both epithelial and endothelial cells (**Table 1**). Although the binding to the pathogen is via surface-exposed protein(s) and the gC1q domain, the adherence and invasion is facilitated by the interaction of C1q collagen region with the host cell-surface receptor, implying that C1q acts as a link between the host and pneumococci (34). Pneumococcal endopeptidase O (PepO) binds strongly to C1q causing activation of classical pathway and consuming the complement components, which allows escape mechanism for Gram-positive species *Streptococcus pneumoniae*. PepO also binds to the C4BP, an inhibitor of the classical pathway (37).

C1q has long been considered a prototypical innate immune molecule with a range of diverse ligands and functions. C1q and its pathophysiological importance can never be over-estimated. C1q involvement in apoptotic cell clearance and its deficiency linked to the development of lupus have been some of the path breaking observations. Its neuroprotective role in clearing protein aggregates in the CNS and perhaps exaggerating neuroinflammation is firmly established. The local synthesis of C1q by immune and non-immune cells and its functions independent of other complement proteins have put the molecule at the forefront of the homeostatic machinery. Its interaction with novel receptors linked with apoptosis induction and aging are exciting areas for further investigation. The roles of C1q in the CNS pathophysiology and development are some of the highlights of complement research in last decade.

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Transcriptional factor PU.1 regulates decidual C1q expression in early pregnancy in human

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C1q is the first recognition subcomponent of the complement classical pathway, which in addition to being synthesized in the liver, is also expressed by macrophages and dendritic cells (DCs). Trophoblast invasion during early placentation results in accumulation of debris that triggers the complement system. Hence, both early and late components of the classical pathway are widely distributed in the placenta and decidua. In addition, C1q has recently been shown to significantly contribute to feto-maternal tolerance, trophoblast migration, and spiral artery remodeling, although the exact mechanism remains unknown. Pregnancy in mice, genetically deficient in C1q, mirrors symptoms similar to that of human preeclampsia. Thus, regulated complement activation has been proposed as an essential requirement for normal successful pregnancy. Little is known about the molecular pathways that regulate C1q expression in pregnancy. PU.1, an Ets-family transcription factor, is required for the development of hematopoietic myeloid lineage immune cells, and its expression is tissue-specific. Recently, PU.1 has been shown to regulate C1q gene expression in DCs and macrophages. Here, we have examined if PU.1 transcription factor regulates decidual C1q expression. We used immune-histochemical analysis, PCR, and immunostaining to localize and study the gene expression of PU.1 transcription factor in early human decidua. PU.1 was highly expressed at gene and protein level in early human decidual cells including trophoblast and stromal cells. Surprisingly, nuclear as well as cytoplasmic PU.1 expression was observed. Decidual cells with predominantly nuclear PU.1 expression had higher C1q expression. It is likely that nuclear and cytoplasmic PU.1 localization has a role to play in early pregnancy via regulating C1q expression in the decidua during implantation.

Keywords: C1q, decidua, pregnancy, trophoblast, stromal cells, pregnancy, implantation, transcription factor

INTRODUCTION

Decidua plays a critical role in accepting the semi-allogenic fetus and protecting it from the mother's immune system during pregnancy (1). Immunological cross-talk between the mother (stromal cells) and the fetus (trophoblasts) takes place in the decidua (2). Several immune and non-immune cells such as macrophages, dendritic cells (DCs), natural killer (NK) cells, T cells, stromal cells, and trophoblasts are involved in the maintenance of early pregnancy at the feto-maternal interface (3). Most of the decidual cells are hematopoietic cells derived from bone marrow (4–7). The process of lineage is tightly regulated by the transcription factors (8, 9). PU.1, an Ets-family transcription factor, was originally shown to be transcriptionally up-regulated in murine erythroleukemia following proviral integration of "spleen focus forming virus" (SFFV) (known as SP11) in humans (10, 11). PU.1 regulates the development and differentiation of myeloid lineage cells (macrophages, DCs, and neutrophils), B cells, NK cells, and T cells (12–15). In addition to being tissue-specific, PU.1 is also known to regulate hematopoiesis (16–18). Recently, PU.1 transcription factor has also been shown to regulate C1q gene expression in macrophages and DCs (19). The strong association between PU.1 and C1q expression raised the possibility that PU.1 may be responsible for C1q expression in the decidua during pregnancy.

C1q is the first recognition subcomponent of the complement classical pathway, which is expressed by the trophoblast and stromal cells in the decidua. Thus, C1q is considered to be involved in promoting interaction between endovascular trophoblast (ET) and endothelial cell. These fetal ET cells invading the decidua synthesize C1q as it differentiates into extravillous trophoblasts (EVT) (20, 21). Moreover, implantation sites in C1q knock-out mice contain reduced number of remodeled vessels and mirror the symptoms of human preeclampsia (22). Decidua from preeclampsia patients has a large number of unremodelled spiral arteries due to defective C1q expression (23). Therefore, C1q appears to regulate a range of functions of decidual cells that are critical for placental development and the absence of C1q leads to the onset of preeclampsia. However, the underlying mechanisms are not fully understood.

Here, we have analyzed human decidua during early pregnancy for the presence and distribution of C1q with the aim

of determining whether PU.1 is involved in decidual regulation of C1q. We show that decidual cells express PU.1 transcriptional factors along with C1q. The present study suggests that localization of PU.1 may be critical for controlling decidual C1q expression.

MATERIALS AND METHODS

CLINICAL SAMPLES

Decidual tissue samples were obtained from ten healthy women aged 20–35 years old who underwent elective vaginal termination of first trimester pregnancy (8–12 weeks). Informed verbal and written consent were obtained from all the subjects. The Domain Specific Review Board of National University Health System, Singapore approved the study. In each case, approximately 2–3 g of tissue were collected. All experiments were repeated at least minimum three times.

IMMUNOHISTOCHEMISTRY

Decidual tissues of approximately 1 cm^2 were rinsed generously in phosphate buffered saline (PBS) to remove any blood clots. Tissues were fixed in 4% paraformaldehyde (PFA) at room temperature for 2–3 days and embedded in paraffin. Immunohistochemistry was carried out as described previously (24). Briefly, 5 μm thick sections of tissues were deparaffinized, and rehydrated in descending ethanol gradient (90, 70, 50, and 25% ethanol). The endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in dark and slides were boiled in sodium citrate buffer using microwave. The sections were permeabilized using 0.1% saponin in order to access the intracellular proteins. Non-specific binding of primary antibodies was blocked using 5% normal goat serum. Tissue sections were probed with rabbit anti-human PU.1 (H-135, Santa Cruz) and incubated overnight at 4°C. Following three washes in PBS, the sections were incubated for 2 h with anti-rabbit IgG secondary antibody conjugated with HRP in dark. Finally, substrate and chromogen (3,3′-diaminobenzidine DAB; Vector) were added to the slides and counterstained with hematoxylin. Primary antibody was replaced with rabbit IgG that served as negative control. The sections were viewed under a Leica DM3000 optical microscope Leica Microsystems), and images were taken using a digital camera (Leica Microsystems) on a computer hard drive.

ISOLATION OF DECIDUAL CELLS, DECIDUAL STROMAL CELLS

The methods were previously described by Singh et al. (24). Briefly, decidual cells were isolated by enzyme treatment and gradient centrifugation. The tissues were finely sliced into small pieces and washed using PBS with constant stirring to remove most of the remaining blood. The tissues were washed with PBS by centrifugation at 400 × *g* for 5 min. The tissues were suspended in RPMI-1640 medium at 37°C and incubated with collagenase type IV, for 1 h at 37°C. Later, centrifugation was carried out at 650 × *g* for 10 min. The supernatant containing released cells were discarded. The pellet was passed through 40 μm filters, washed three times in PBS by centrifugation, and left overnight to recover at 4°C with RPMI-1640 containing 10% v/v heat-inactivated FCS. Next day, the cells were collected after washing in PBS by centrifugation.

Decidual cells at the concentration of 1×10^6 cells/ml were incubated in 12 well-plates (Nunc) with coverslips for 24 h in compete RPMI medium containing with 2% heat-inactivated FCS to allow stromal cells to adhere to the wells. After overnight incubation, supernatant containing non-adherent hematopoietic cells were discarded leaving 98% pure adherent stromal cells. Proliferating decidual stromal cells (DSCs) outgrew other possible contaminating cells, and thus, pure populations of cultured stromal cells were obtained. The supernatant was discarded; the cells were washed carefully, and then re-suspended in PBS for further analysis.

The concentration of decidual cells were adjusted to 0.050×10^4 cells/200 μl using PBS. Slides were cytospun (700 rpm for 3 min) using cytocentrifuge (Shandon cytospin III) and air dried at room temperature. Slides were stored at room temperature overnight, or alternatively, used immediately for staining.

POLYMERASE CHAIN REACTION

Total RNA was extracted from decidual cells and DSCs using Macherey-Nagel RNA isolation kit according to the manufacturer's protocol. The concentration of the extracted RNA was determined by absorbance at 260 nm and the purity was estimated via A260/A280 ratio with Nanodrop spectrophotometer ND-1000 (Thermo Scientific). To evaluate the quality of RNA extracted, 1 μg of total RNA were reverse transcribed (Superscript II reverse Transcriptase kit, Invitrogen) and amplified (GoTaq PCR kit, Promega) with primers (custom-made by AIT biotech, Singapore) for the housekeeping gene GAPDH. Human macrophages cells, isolated as previously described by Cao et al. (25), were used as internal control. The PCR amplified products were separated on 1.5% agarose gel containing gel green along with 100-bp ladder (Fermentas) for visualization. GAPDH expression was used an endogenous reference gene and analyzed in parallel in all samples.

PRIMERS

For PU.1, the primers used were forward, 5′-AGAGCCATAGCGAC CATTA-3′; and reverse, 5′-TATCGAGGACGTGCATCT-3′ (product, 162 bp). For GAPDH, the primers were forward, 5′-CGGAGT CAACGGATTTGGTCG-3′; and reverse, 5′-TCTCGCTCCTGGAA GATGGTGAT-3′ (product, 225 bp).

INTRACELLULAR SINGLE IMMUNOFLUORESCENCE STAINING

Decidual cells on cytospin slides were fixed with 4% PFA. Cells were permeabilized with 0.1% saponin in PBS and then incubated with rabbit anti-human PU.1 (H-135) primary antibody in 5% BSA in PBS on ice, followed by staining with relevant goat-anti-rabbit FITC. Cells were washed in PBS, and then mounted using Vectashield with 4′, 6-diamidino-2-phenylindole (DAPI). The cellular localization of PU.1 with FITC was determined using Axiolmager Z1 fluorescent microscope. The images were captured and recorded with digital camera (Zeiss Axiocam MRc). Cells incubated with Isotype IgG were used as non-specific controls.

INTRACELLULAR SINGLE IMMUNOFLUORESCENCE STAINING FOR CONFOCAL MICROSCOPY

Decidual cells on cytospin slides were fixed with 4% PFA. The cells were permeabilized with 0.1% saponin in PBS and then incubated with rabbit anti-human PU.1 (H-135) primary antibody in 5% BSA on ice, followed by staining with relevant goat-anti-rabbit IgG conjugated with Cy3. Cells were washed in PBS, and then mounted using Vectashield with DAPI. The cellular localization of

goat-anti-rabbit Cy3 fluorescence showing the presence of PU.1 in decidual cells was determined using confocal microscope with 60× oil objective and the images were digitally recorded on a computer hard drive. Cells incubated with Isotype IgG were used as non-specific controls.

DOUBLE IMMUNOFLUORESCENCE STAINING

Vimentin/PU.1

Decidual stromal cells on cytospin slides were incubated with mouse anti-human vimentin (1:150; Dako). After washing in PBS, slides were fixed in 4% PFA, and permeabilized in 0.1% saponin in PBS for 10 min. The slides were incubated with rabbit anti-human PU.1 H-135 (1:600; Santa Cruz). After washing with PBS, the slides were incubated with goat-anti-mouse FITC (3:100) and goat-anti-rabbit Cy3 (1:200) conjugates.

CK-7/PU.1

Decidual cells on cytospin slides were fixed with 4% PFA and permeabilized with 0.1% saponin in PBS for 10 min. The slides were incubated with mouse anti-human CK-7 (1:100; Abcam) and rabbit anti-human PU.1 H-135 (1:600, Santa Cruz). After washing with PBS, the slides were incubated with goat-anti-mouse Cy3 (1:200) and goat-anti-rabbit FITC (3:100) conjugates.

C1q/PU.1

Decidual cells on cytospin slides were fixed with 4% PFA and permeabilized with 0.1% saponin in PBS for 10 min. The slides were incubated with mouse anti-human C1q (1:200, Abcam) and rabbit anti-human PU.1 H-135 (1:600, Santa Cruz). After washing with PBS, the slides were incubated with goat-anti-mouse Cy3 (1:200) and goat-anti-rabbit FITC (3:100) conjugates. As controls, we analyzed the expression of the same in RAW cells using identical conditions.

All primary and secondary antibodies were diluted in 5% BSA and incubated 1 h in ice. The slides were finally mounted using Vectashield DAPI (Vectastain, Vector Laboratories). The cellular localizations of various proteins were examined using Zeiss Axiolmager Z1 fluorescent photomicroscope. The images were captured and recorded using digital camera (Zeiss Axiocam MRc). Cells incubated with Isotype IgG were used as non-specific controls.

RESULTS

IMMUNOHISTOCHEMICAL ANALYSIS OF PU.1

First, we investigated the presence of PU.1 in paraffin-embedded decidual sections obtained from women who underwent elective termination of pregnancy. As shown in **Figures 1A,B**, PU.1 immunostaining was intense in decidual tissues. PU.1 expression was consistent between decidual samples (case to case), but varied field to field. In few areas, weak and diffused staining was observed (**Figure 1D**). Notably, some cells did not stain for PU.1 suggesting that not all decidual cells express PU.1. We analyzed the expression of PU.1 in decidua obtained from five different subjects. Immunoreactivity was absent in control slides treated with rabbit IgG. Taken together, these results suggested that not all, but some, decidual cells synthesize PU.1 transcription factor.

EXPRESSION OF PU.1 IN EARLY HUMAN DECIDUA ANALYZED BY RT-PCR

To further confirm the specificity of the antibody used in immunohistochemistry, we examined the expression of PU.1 mRNA in early human decidua. Using a fragment of 162 bp corresponding to human PU.1 proto-oncogene transcript variant 1 was amplified by RT-PCR. The PU.1 mRNA was easily detected in the decidual cells (**Figure 2A**). Since decidual cells expressed PU.1, we tested whether DSCs also expressed PU.1 mRNA. Thirty-five cycles of PCR amplification after the reverse transcriptase reaction yielded

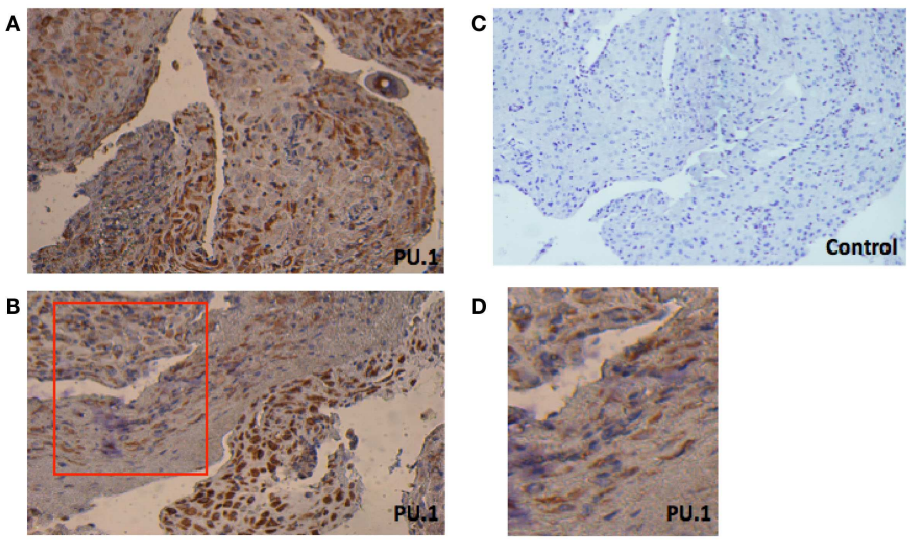

FIGURE 1 | Immunohistochemical localization of PU.1 in early pregnancy decidual tissue obtained from elective termination of pregnancy. The tissues were stained by DAB and counterstained with hematoxylin, as described in Section "Materials and Methods." The presence of PU.1 transcription factor is stained brown and hematoxylin in blue **(A,B)**. Control sections pre-incubated with rabbit IgG were uniformly negative **(C)**. The red box in **(B)** indicates enlarged image as seen in **(D)**. The experiment was performed using decidual tissues from five subjects. Original magnification 40×.

products derived from PU.1 mRNA in both decidual cells and DSCs (**Figure 2A**). The experiment was repeated with four different decidual samples. GAPDH primer pair was used as an internal control (**Figure 2B**) in addition to human macrophages cells that were used as a positive control (**Figure 2A**). The results confirmed the moderate-to-strong expression of PU.1 transcription factor at transcriptional as well as and translational level in decidual cells and DSCs.

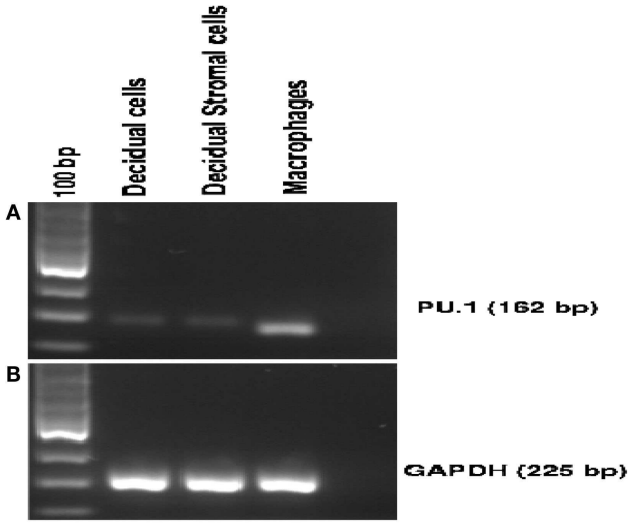

FIGURE 2 | Expression of PU.1 transcription factor by human first trimester decidua. Decidual cells and decidual stromal cells isolated from human decidua were analyzed for PU.1 (162 bp) expression. For RT-PCR, 1 μg of total RNA isolated from decidual cells, decidual stromal cells, and human macrophages was used as a template. PU.1 cDNA from decidual cells, decidual stromal cells, and human macrophages were amplified using specific primer set mentioned in Section "Materials and Methods" **(A)**. GAPDH was amplified to serve as an internal control **(B)**. Human macrophage cells served as a positive control **(A)**. All four decidual samples were positive for PU.1 expression.

CELLULAR LOCALIZATION OF PU.1 TRANSCRIPTION FACTOR IN EARLY HUMAN DECIDUA

To further characterize the cellular localization of PU.1 transcription factor, decidual cells were cytospun on coverslips, fixed, permeabilized, and immunostained with rabbit anti-human PU.1 (H-135) antibody. The cells were then mounted with DAPI. Transcription factors PU.1 has previously been localized in the nucleus (26). As expected, nuclear expression of PU.1 was detected in the decidual cells, although cytoplasmic staining of PU.1 was also observed (**Figure 3**). The immunostaining of decidual cells for PU.1 indicated the presence of PU.1 transcription factor in differential locations in various decidual cells.

EXAMINATION OF CELLULAR LOCALIZATION OF PU.1, TRANSCRIPTION FACTOR IN EARLY HUMAN DECIDUA BY CONFOCAL MICROSCOPY

To rule out the possibility that localization of PU.1 in the cytoplasm is not due to goat-anti-mouse FITC coupled secondary antibody, we stained the decidual cells with rabbit anti-human PU.1 (H-135) antibody after permeabilization. Instead of FITC, goat-anti-rabbit Cy3 conjugate was used, counterstained with DAPI, and then analyzed by confocal microscope. As shown in **Figure 4**, PU.1 was localized in the cytoplasm of decidual cells. Although the reason for differential distribution of PU.1 in decidual cells is unclear and since PU.1 is well known for its role in hematopoiesis, it is likely that its expression is associated with different cell-cycle stages in early stages of pregnancy.

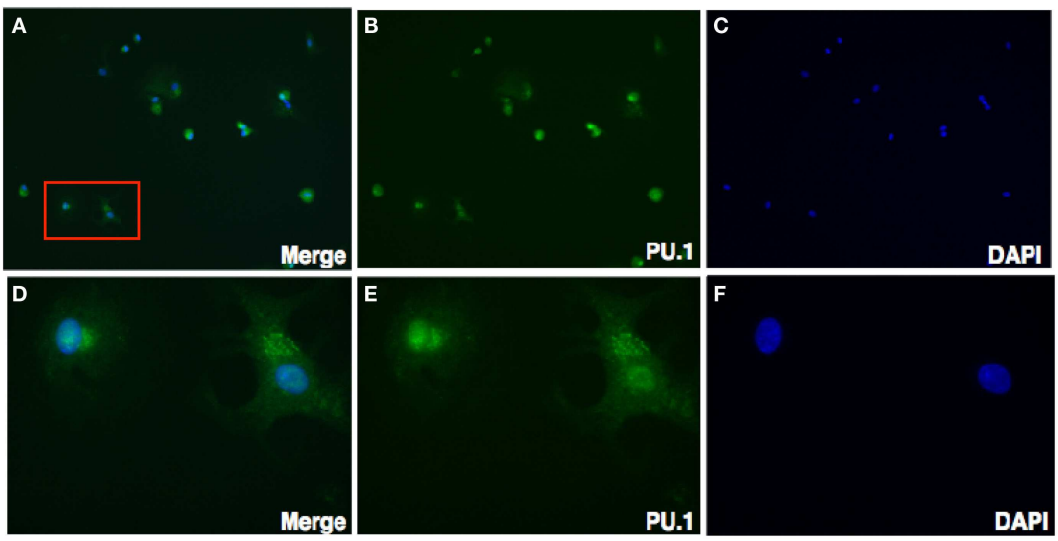

FIGURE 3 | Immunofluorescence staining for PU.1 expression in first trimester decidua. Decidual cells cytospun were fixed, permeabilized, immunostained with rabbit anti-human PU.1 antibody, and then probed with goat-anti-rabbit FITC. After counterstaining with DAPI, analysis was carried out using Axiolmager Z1 fluorescent microscope. Decidual cells stained with DAPI, PU.1 and overlay at 40× magnifications **(A–C)**. Cells in red box **(A)** indicate the same at higher magnification, 100× oil immersion stained with DAPI, PU.1, and overlay in **(D–F)**.

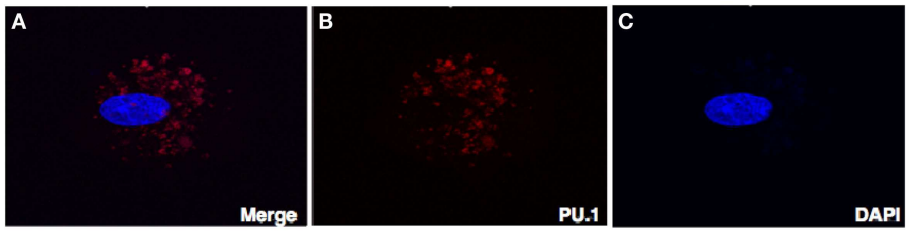

FIGURE 4 | Confocal microscopy of the decidual cells expressing PU.1 transcription factor in the cytoplasm and nucleus. Decidual cells fixed, permeabilized, immunostained with rabbit anti-human PU.1 antibody and then probed with goat-anti-rabbit Cy3 **(B)**, counterstained with DAPI **(C)**, and analyzed by confocal microscope **(A)**. Original magnification 60×.

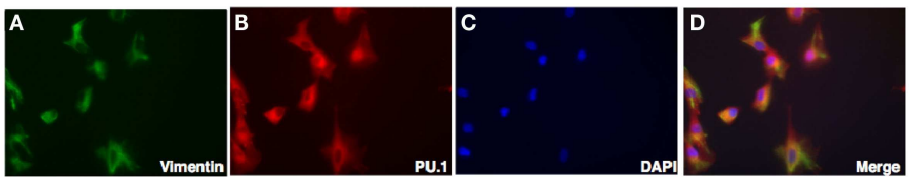

FIGURE 5 | Double immunofluorescence staining of PU.1 and stromal cells in first trimester decidua. Pure population of stromal cells over coverslips was immunostained with mouse anti-human vimentin and rabbit anti-human PU.1 antibody. This was followed by probing with anti-mouse FITC and anti-rabbit Cy3, counterstained with DAPI and analyzed by Axiolmager Z1 fluorescent microscope at 40× magnifications **(A–D)**.

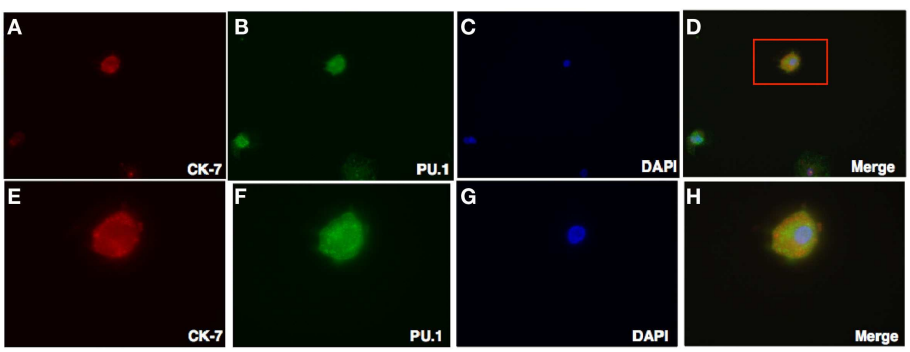

FIGURE 6 | Double Intracellular immunofluorescence staining of PU.1 and trophoblasts in first trimester decidua. Decidual cells cytospun were fixed, permeabilized, and then immunostained with mouse anti-human CK-7 and rabbit anti-human PU.1 antibodies. This was then probed with goat-anti-mouse Cy3 and anti-rabbit FITC conjugates, counterstained with DAPI and analyzed by Axiolmager Z1 fluorescent microscope at 40× magnifications **(A–D)**. Cells in red box **(D)** indicate the same at higher magnification, 100× oil immersion stained with CK-7, PU.1, DAPI, and overlay in **(E–H)**.

EXPRESSION OF PU.1 TRANSCRIPTION FACTOR BY DSCs AND TROPHOBLASTS

Prompted by the PCR data, to gain insight into the intracellular localization of PU.1, we investigated stromal cells and trophoblasts by dual immunofluorescence against vimentin and cytokeratin-7. Majority of DSCs were found to express PU.1 either in the nucleus or in the cytoplasm (**Figure 5**). On the other hand, in trophoblasts cytoplasmic expression of PU.1 was observed. Remarkably, PU.1 positive staining was seen in very few trophoblasts (**Figure 6**). Thus, very few trophoblasts express PU.1, which mainly showed cytoplasmic staining. The presence of PU.1 with differential sub-cellular localization within the pure population of DSCs and trophoblasts confirms that PU.1 is definitely involved in the regulation of DSCs and trophoblasts. Furthermore, difference in the PU.1 staining pattern could possibly be due to its involvement in differentiation of DSCs and trophoblasts, thereby preparing for decidualization.

C1q PRODUCED BY DECIDUAL CELLS EXPRESSING NUCLEAR PU.1

We have shown via intracellular staining that DSCs and trophoblasts produce C1q (**Figure 7**), consistent with earlier studies (20, 21). To determine if PU.1 expressing cells also produced C1q, dual immunofluorescence staining was employed. Recent studies have indicated that PU.1 transcription factor regulates C1q expression in macrophages and DCs (19). Having found that PU.1 was widely distributed in the decidual cells (**Figures 1**

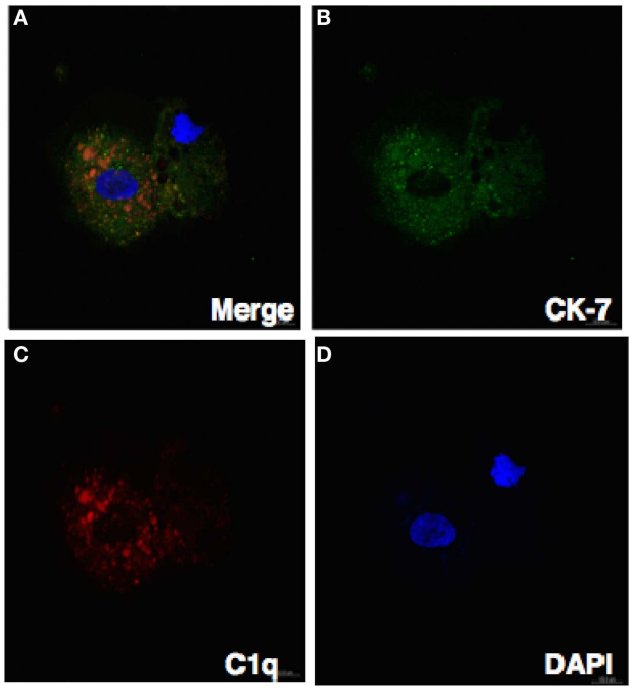

FIGURE 7 | Confocal image showing the trophoblast expressing C1q. Decidual cells cytospun were fixed, permeabilized, immunostained with mouse anti-human CK-7 and goat-anti-human C1q antibodies, and then probed with donkey anti-mouse FITC and donkey anti-goat Cy3 conjugates, counterstained with DAPI and analyzed by confocal microscope **(A–D)**. Original magnification 60× oil immersion.

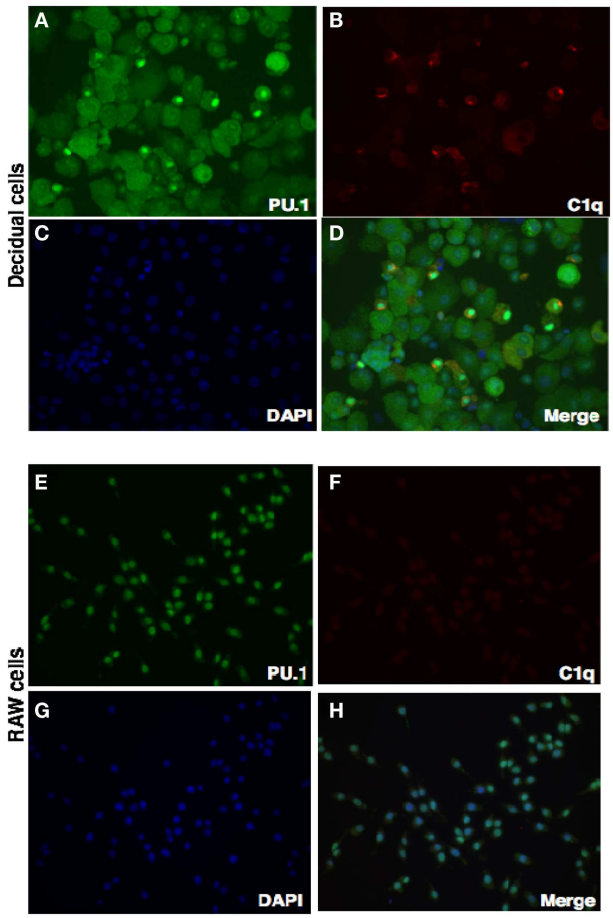

FIGURE 8 | Double intracellular immunofluorescence staining of PU.1 and C1q in first trimester decidua. Decidual cells cytospun were fixed, permeabilized, and immunostained with mouse anti-human CK-7 and rabbit anti-human PU.1 antibodies. This was then probed with goat-anti-mouse Cy3 and anti-rabbit FITC conjugates, counterstained with DAPI and analyzed by Axiolmager Z1 fluorescent microscope **(A–D)**. RAW cells were used as controls treated and analyzed as decidual cells **(E–H)**. Original magnification for decidual cells and RAW cells are 20× magnifications.

and **2**), both PU.1 and C1q were detected in the decidual cells. Interestingly, decidual cells with significantly high levels of C1q expression showed PU.1 exclusively in the nucleus. They showed more intense staining in the nucleus but cytoplasmic staining was is also observed. (**Figures 8A–D**) when compared to RAW cells (**Figures 8E–H**). This indicates the close relationship between C1q and nuclear PU.1 gene expression.

DISCUSSION

In this study, we have investigated the relationship between PU.1 and C1q, and their likely regulation in early human decidua. We show that PU.1 is abundantly expressed in the decidua cells. To our knowledge, this is the first demonstration of the cellular localization of transcription factor PU.1 in the early human decidua. Nuclear PU.1 is likely to be a critical regulator of C1q expression in decidual cells based on the immunofluorescence staining pattern. PU.1 is an E26 transformation-specific (Ets) family transcription factor specifically involved in early and late stages of myeloid lineage cell differentiation (27). PU.1, known as SFFV proviral integration site 1 (SPi1) in humans, consists of an N-terminal transcriptional domain (100 amino acids) and a C-terminal DNA-binding domain (112 amino acids). Its gene is located on the shorter arm of chromosome 11p11.2 (28). The selected H-135 antibody raised against PU.1 recognizes amino acid 1-135 at the N-terminus of PU.1. Use of this antibody has previously established that PU.1 regulates C1q gene expression in macrophages and DCs (19).

Immunohistochemical analysis of paraffin-embedded first trimester decidua with rabbit anti-human PU.1 (H-135) showed a wide distribution of transcriptional factor PU.1. Overall, a strong immunoreactivity for PU.1 was observed (**Figures 1A,B**). To further validate this, we analyzed the PU.1 gene expression by RT-PCR in the first trimester decidua. Initially, we performed RT-PCR with decidual cells that suggested local synthesis of PU.1. Since DSCs are a key component of human decidua, we investigated the ability of DSCs to synthesize and secrete PU.1 (**Figure 2A**). Analysis of stromal cells also revealed the presence of PU.1, suggesting that decidual cells, in particular DSCs, expressed PU.1 at mRNA and protein level.

Next, we characterized the sub-cellular localization of PU.1 in decidual cells. Isolated decidual cells were cytospun, fixed,

permeabilized, and immunostained with rabbit anti-human PU.1 (H-135) antibody. Surprisingly, the staining of the decidual cells with PU.1 indicated its differential locations among different cells within the decidua. Certain cells showed PU.1 expression in the nucleus whereas others showed immunofluorescence staining in the cytosol; some cells did not show PU.1 expression at all (**Figure 3**). This result was unexpected as the localization of transcription factor is usually within the nucleus. Because PU.1 was observed in the cytoplasm, we wanted to verify our speculation that the cellular localization of PU.1 was not due to the FITC conjugated secondary antibody. Therefore, we analyzed the cellular localization of PU.1 with Cy3 conjugated secondary antibody using confocal microscopy (**Figures 4A–C**). The observations from immunofluorescence staining by Axiolmager Z1 fluorescent and confocal microscopy demonstrated that PU.1 expression was present in a restrictive manner at specific locations within the decidual cells. Further studies on cellular localization of PU.1 in decidua will allow a better understanding of the functional significance of PU.1 in pregnancy.

To study the cell-type specific cellular localization of PU.1 in the first trimester decidua, stromal cells, and trophoblasts were stained with PU.1 and their respective markers vimentin and cytokeratin-7. Both stromal cells and trophoblasts were capable of synthesizing PU.1. Notably, all stromal cells were PU.1 positive (**Figure 5**) while very few trophoblasts revealed PU.1 expression (data not shown). The detection of increased nuclear PU.1 expression in stromal cells when compared to trophoblasts is puzzling. Furthermore, we observed that the PU.1 is expressed to a significantly higher level in the stromal cells than in the trophoblasts from first trimester decidua (**Figures 5** and **6**). Decidual leukocytes are proposed to trigger the endometrial stromal cell differentiation that encourages invasion of trophoblast by secreting IL-17 (29, 30). This cross-talk between stromal cells and trophoblasts is thus considered to regulate the immune milieu at feto-maternal interface for successful implantation.

To the best of our knowledge, this is the first study that addresses the expression of PU.1 in early human decidua. PU.1 not only regulates cellular process such as proliferation and differentiation but its relative expression levels are myeloid-specific regulating hematopoietic lineage commitment (13, 31–33). Our data strongly suggest that PU.1 is likely to be involved in stromal cell differentiation. The finding that few trophoblasts from first trimester decidua express PU.1 may pertain to special type of trophoblasts such as invasive EVT or syncytio-trophoblasts, which are further differentiating while invading the maternal decidua. Together, the differential expression patterns of PU.1 on stromal cells and trophoblasts reflect the differences in the requirement for specific functional roles in different cells within the decidua.

Previous studies have shown that first trimester decidual cells produce C1q and likely to play a role in trophoblast invasion and placental development (20, 21). Using immunofluorescence staining, we also show here the expression of C1q in both trophoblasts and stromal cells (**Figure 7**; Madhukaran et al., submitted). Next, we examined the ability of PU.1 to regulate C1q expression since PU.1 has previously been shown to regulate C1q gene expression in macrophages and DCs (19). To evaluate the contribution of PU.1 in decidual C1q expression, we immunostained decidual cells for PU.1 and C1q. Strong staining for C1q and PU.1 was observed in the decidual cells when compared to RAW cells (**Figure 7**). Moreover, we observed that PU.1 expression was confined to the nucleus in decidual cells that had significant increase in C1q expression (**Figures 7A–D**). The immunofluorescence staining data indicate, for the first time, the presence of nuclear PU.1 location in decidual cells expressing C1q. In contrast, cytoplasmic localization of PU.1 staining was also detected in cells with no or less C1q expression. The presence of nuclear PU.1 in decidual cells expressing C1q suggests that PU.1 may be regulating the production of decidual C1q. We have recently found high level of C1q expression in all stromal cells, while very few trophoblasts exhibit C1q expression (Madhukaran et al., submitted). It would be of interest to study the expression levels of both PU.1 and C1q in stromal cells and trophoblasts associated with normal pregnancy and preeclampsia in humans.

If PU.1 and C1q are normally expressed in early human decidua, their relative levels could regulate trophoblast and stromal cell specific lineage differentiation during early implantation. The association of PU.1 and C1q expression in first trimester human decidua and the exact role may be fundamental to our understanding of the lineage commitment of PU.1 that regulates C1q expression during normal pregnancy. Taken together, our data suggest that decidual cells transcribe and translate PU.1 and it is selectively localized in the stromal cells and trophoblasts. Additional studies aimed at understanding the PU.1 regulation of C1q gene expression in decidua during normal pregnancy are required. It also raises the possibility of understanding the functional implications of cytoplasmic PU.1 in early human decidua during implantation of normal as well as complicated pregnancies such as preeclampsia.

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Anti-C1q autoantibodies, novel tests, and clinical consequences

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Although anti-C1q autoantibodies have been described more than four decades ago a constant stream of papers describing clinical associations or functional consequences highlights that anti-C1q antibodies are still hot and happening. By far the largest set of studies focus on anti-C1q antibodies is systemic lupus erythematosus (SLE). In SLE anti-C1q antibodies associate with involvement of lupus nephritis in such a way that in the absence of anti-C1q antibodies it is unlikely that a flare in nephritis will occur. Anti-C1q antibodies occur in several autoimmune conditions but also in healthy individuals. Although considerable progress has been made in the understanding of how anti-C1q antibodies may contribute to tissue injury there is still a lot to learn about the processes involved in the breaking of tolerance to this protein. There has been considerable improvement in the assays employed to test for the presence of anti-C1q antibodies. Hopefully with these new and standardized assays at hand larger clinical association studies will be conducted with independent replication. Such large-scale studies will reveal the true value of clinical testing for anti-C1q autoantibodies in several clinical conditions.

Keywords: C1q, complement, autoantibody, diagnosis, SLE

INTRODUCTION

Of all the autoantibodies that target complement proteins, anti-C1q autoantibodies have received most attention (Trouw et al., 2001; Norsworthy and Davies, 2003). C1q, the initiation molecule of the classical pathway of complement activation, has a unique capacity to bind to the Fc tail of subclasses of IgG and IgM antibodies (Daha et al., 2011). C1q does so only when at least two IgG molecules are spatially oriented in such a way that they can simultaneously interact with one C1q molecule as for example in an immune-complex (Cooper, 1985). Alternatively, C1q can bind to a single IgM molecule in a "staple" like configuration (Feinstein et al., 1971). The binding of C1q to non-aggregated IgG molecules or fluid-phase IgM is weak. The fact that C1q can bind IgG in immune complexes can be considered as both a blessing and a curse. The blessing lies in the fact that the identification of anti-C1q antibodies was a consequence of studies on size fractionations of immune complexes that could bind to C1q. In these studies it was subsequently discovered that in systemic lupus erythematosus (SLE) patients next to the high molecular weight fractions also low-molecular weight fractions contained immunoglobulins that could bind to C1q (Agnello et al., 1971) (**Table 1**). In the following years these low-molecular weight fractions were further identified as monomeric, non-complexed IgG molecules that specifically interacted with the collagen-like tail of the C1q molecule (Uwatoko et al., 1984, 1987; Antes et al., 1988). The curse lies in the fact that special care has to be taken to discriminate between IgG in immune complexes binding to C1q and anti-C1q autoantibodies binding to C1q (Kohro-Kawata et al., 2002). This problem can be overcome by adding 1 M NaCl to the incubation buffer in the assay. The low-avidity interactions between the C1q globular head domains with the CH2 domains of IgG Fc tails is completely disrupted in these conditions, whereas the high avidity binding of anti-C1q autoantibodies to the collagen-like tail of C1q is kept intact (Kohro-Kawata et al., 2002). It is currently not known to what extend the high salt conditions may lead to an underestimation of low-avidity anti-C1q autoantibodies.

ASSAYS TO DETECT ANTI-C1Q AUTOANTIBODIES

Over time several assays have been developed to detect anti-C1q autoantibodies both in humans and in experimental animal models. The first assays employed a direct coating of intact C1q, which necessitated the use of high salt conditions to discriminate between immune-complex binding and anti-C1q autoantibody binding (Kohro-Kawata et al., 2002). Already early in the history of anti-C1q autoantibodies it was discovered that the majority of these autoantibodies is directed against the collagen-like part of the C1q molecule (Antes et al., 1988). From equilibrium studies and from the observation that anti-C1q antibodies can be found in the presence of freely circulating C1q it was argued that anti-C1q antibodies may interact with epitopes that are not exposed in C1q in fluid phase (Golan et al., 1982). Later these arguments were supported by elegant studies using phage display technology generated Fab fragments that only interacted with solid-phase C1q (Schaller et al., 2009). Next, assays have been developed that utilized only the C1q collagen-like region, generated by enzymatic digestions as antigen (Antes et al., 1988; Wener et al., 1989). This eliminated the need to use high-ionic strength buffer. A recent paper reports on the use of peptides derived from C1q that have interesting properties to detect a major linear epitope in a high percentage of the patients in the absence of high-ionic strength

Table 1 | History of anti-C1q antibodies.

Year	Milestone	Reference
1971/88	Identification of C1q as the target of autoantibodies	Agnello et al. (1971), Antes et al. (1988)
1982	C1q in solid-phase exposes neo-epitopes	Golan et al. (1982)
1984	Identification of the collagen-like stalk as the main binding site of anti-C1q antibodies	Uwatoko et al. (1984, 1987)
1987	Anti-C1q associates with the occurrence of LN	Wener et al. (1987); reviewed a. o. Trendelenburg (2005)
1991	C1q and anti-C1q are enriched in the glomeruli of LN	Uwatoko et al. (1991)
1996	Identification of anti-C1q in mice	Hogarth et al. (1996)
1993	Anti-C1q also present in healthy population and increase with age	Siegert et al. (1993)
2004	Experimental evidence on how anti-C1q can be pathogenic to the kidney in LN but not in healthy individuals	Trouw et al. (2004a)
2007	Identification of anti-C1q antibodies that target the globular heads	Tsacheva et al. (2007)

Table 2 | Overview of commercially available assays for the detection of anti-C1q antibodies.

Trade name (P/N)	Company	Technology	Reference
Anti-C1q autoantibody ELISA (EK-AC1QA)	Buehlmann Laboratories AG	ELISA	Trendelenburg et al. (2006), Potlukova et al. (2008), Meyer et al. (2009), Julkunen et al. (2012)
IMTEC-anti-C1q-antibodies (ITC59033)	IMTEC	ELISA	Cai et al. (2010)
QUANTA lite anti-C1q (704565)	INOVA Diagnostics	ELISA	Akhter et al. (2011)
Anti-C1q (ORG 549)	Orgentec	ELISA	Heidenreich et al. (2009), Julkunen et al. (2012)
Anti-C1q (ORG 249)	Orgentec	Alegria	NA

buffer (Vanhecke et al., 2012). In contrast to the assays reported before that anti-C1q antibodies only target the collagen-like region of C1q in 2007 it was discovered that there are also antibodies that specifically target the globular head regions of C1q (Tsacheva et al., 2007).

To study anti-C1q antibodies in experimental animal models, assays were developed that used coating of purified mouse C1q and high salt conditions similar to the human situation (Hogarth et al., 1996; Trouw et al., 2003). Next, in order to circumvent the purification of mouse C1q, an assay was developed which employed a coating of C1q binding peptides, that captured C1q from $Rag^{-/-}$ serum, as the antigenic entity for the anti-C1q ELISA (Trouw et al., 2004a,b).

Several commercial assays are available for the detection of anti-C1q antibodies (see **Table 2**). Those assays are mostly based on the ELISA technology and are marketed by Bühlmann Laboratories AG (CH-4124 Schönenbuch, Switzerland), IMTEC (HUMAN, Wiesbaden, Germany), Orgentec (Mainz, Germany), and INOVA Diagnostics (San Diego, CA, USA). The ALEGRIA system is a semi-automated assay system based on patient specific modified microtiter plate strips. Some of the anti-C1q antibody assays have been used in clinical studies (Trendelenburg et al., 2006; Potlukova and Kralikova, 2008; Heidenreich et al., 2009; Meyer et al., 2009; Cai et al., 2010; Akhter et al., 2011; Julkunen et al., 2012). Until today, none of the anti-C1q antibody assays achieved clearance by the Food and Drug Administration (FDA) due to the lack of prospective studies. In addition, systematic studies comparing anti-C1q antibody assays from different companies are missing.

OCCURRENCE OF ANTI-C1Q AUTOANTIBODIES AND CLINICAL ASSOCIATIONS

Over the past four decades anti-C1q autoantibodies have been studied in a wide variety of autoimmune and renal conditions as well as in infectious diseases (Trendelenburg, 2005). In the healthy population the prevalence of anti-C1q autoantibodies ranges between 2 and 8% (Wener et al., 1989; Siegert et al., 1992a; Trendelenburg et al., 1999; Horvath et al., 2001a; Potlukova et al., 2008) and increases with age (Siegert et al., 1993). Hypocomplementemic Urticarial Vasculitis Syndrome (HUVS) represents the clinical condition with the highest percentage of anti-C1q positivity; 100% (Wisnieski and Jones, 1992). Other conditions characterized by high anti-C1q antibody prevalence are, mixed connective tissue disease (94%), Felty's syndrome (76%), and SLE (30–60%) (Siegert et al., 1992a; Trendelenburg, 2005; Potlukova et al., 2008; Sinico et al., 2009). The occurrence of anti-C1q autoantibodies was shown to have familial clustering, indicating that there is a genetic risk factor that together with environmental cues may precipitate the production of these antibodies (Hunnangkul et al.,

2008). Anti-C1q autoantibodies have also been described to occur in infectious diseases although at a frequency of around, for example 13% of HIV infected individuals vs. 5% in healthy controls (Prohaszka et al., 1999) or up to 26% in patients suffering from hepatitis C virus infection as compared to 10% of healthy controls (Saadoun et al., 2006).

Especially the association between anti-C1q antibodies and renal involvement in SLE has received much attention (Seelen et al., 2003). Correct diagnosis of a flare of lupus nephritis (LN) still represents an important challenge. Serological identification of a flare would be preferred over repeated renal biopsy. Although several anti-nuclear antibodies are associated with renal involvement and active disease (Heidenreich et al., 2009), the presence of anti-C1q antibodies either alone or in combination with other serological markers is superior to predict/correlate with active LN as reviewed before (Trendelenburg, 2005; Sinico et al., 2009). Several studies provide evidence that anti-C1q autoantibodies are superior to other serological markers in identifying a flare of LN (Mok et al., 2010; Akhter et al., 2011). However, other studies indicate that combinations of anti-C1q antibodies with other serological markers are superior to anti-C1q antibodies alone (Matrat et al., 2011; Julkunen et al., 2012; Yang et al., 2012). Especially striking is the strong negative predictive value of anti-C1q testing for LN. In the absence of anti-C1q autoantibodies it is very unlikely that a patient with LN will develop a flare (Trendelenburg et al., 1999, 2006; Meyer et al., 2009; Mok et al., 2010; Matrat et al., 2011; Moura et al., 2011). As many of these studies report on rather small patient populations from very diverse ethnic backgrounds ranging from Brazil (Moura et al., 2011), China (Zhang et al., 2011), India (Pradhan et al., 2012), and Egypt (ElGendi and El-Sherif, 2009) it is likely that considerable variation exists in the strength at which anti-C1q antibodies are associated with and is predictive for LN flares. Several of the larger studies from Europe and Hong Kong point in the same direction (Moroni et al., 2001; Mok et al., 2010; Julkunen et al., 2012) and also a recent meta-analysis confirmed the diagnostic value of serum anti-C1q antibodies for LN (Yin et al., 2012).

Whether or not anti-C1q antibodies are also associated with the disease activity of LN remains to be established as currently there is no consensus on this issue (Horvath et al., 2001b; Grootscholten et al., 2007; Julkunen et al., 2012). One mechanism to clear anti-C1q autoantibodies from the circulation is to use immunoabsorption on C1q-columns (Hiepe et al., 1999). This method depleted next to circulating immune complexes also anti-C1q autoantibodies and was shown to be beneficial in SLE patients (Berner et al., 2001; Pfueller et al., 2001).

ANTI-C1Q ANTIBODIES AS PART OF MULTIPLEX TECHNOLOGIES

With the emerging availability of multiplex technologies for the detection of autoantibodies and other serological markers, the combination of anti-C1q antibodies with other markers is facilitated (Papp et al., 2012). Recent studies have demonstrated that biomarker profiles have the potential to improve the diagnosis of SLE (Kalunian et al., 2012).

This is important also in the light of new treatment opportunities for SLE (Stohl and Hilbert, 2012) and personalized medicine which seems to be just around the corner (Kalunian and Joan, 2009). The ultimate goal is to develop a panel of serological markers that are able to predict SLE flares which then can be prevented by initiating the appropriate treatment. Especially life threatening complications such as kidney failure and transplantation should be prevented. Recently it has been described that anti-chromatin (anti-nucleosome) antibodies are a promising serological marker to help to predict the need for kidney transplantation (Stinton et al., 2007). Whether testing for anti-C1q antibodies in a multiplex setting has the potential to contribute to the improved management of LN patients remains a matter of further research.

PATHOGENIC CONSEQUENCES OF ANTI-C1Q AUTOANTIBODIES

Several studies have addressed the mechanisms by which anti-C1q autoantibodies may contribute to tissue damage, especially in LN. Immune complexes eluted from affected glomeruli of human patients and experimental animals revealed that there is a strong enrichment of anti-C1q antibodies and that this deposition seemed to occur via solid-phase C1q (Uwatoko et al., 1991; Mannik and Wener, 1997; Trouw et al., 2004b). Next to the previously mentioned clinical association studies also *in vitro* and *in vivo* animal studies have been performed (Siegert et al., 1992b; Hogarth et al., 1996; Trouw et al., 2004a,b; Bigler et al., 2011). Several of the mouse models of lupus are characterized by a progressive autoimmune disease in which autoantibodies are generated, immune complexes are formed followed by the occurrence of severe glomerulonephritis. Depending on the mouse model these autoimmune phenomena may evolve in different degrees of severity and at different ages. Using MRL/lpr, BXSB, and NZB/W mice, with a severe lupus phenotype, it was demonstrated that anti-C1q autoantibodies are also present in mice and that an increase in the titer of anti-C1q antibodies are associated with the onset of nephritis (Hogarth et al., 1996; Trouw et al., 2004b). Using a different model, using MRL/MpJ$^{+/+}$ mice with a less severe lupus phenotype, it was concluded that glomerulonephritis may also occur in the absence of anti-C1q antibodies (Bigler et al., 2011). In a more experimental setting, injection of rabbit anti-mouse C1q antibodies resulted in immune-complex deposition of C1q and anti-C1q antibodies but the limited degree of deposition was insufficient to induce glomerulonephritis (Trouw et al., 2003). However, injection of mouse anti-mouse C1q autoantibodies into animals that have C1q containing immune complexes in the glomeruli, resulted in strong glomerulonephritis (Trouw et al., 2004a). Collectively these data indicate that anti-C1q antibodies can be present in healthy subjects (mouse or human) which might induce limited deposition in the kidney but no nephritis. Only in the presence of C1q containing immune complexes in the kidney, anti-C1q autoantibodies will amplify the local complement activation and cellular influx resulting in glomerulonephritis. A similar process may also be operational in post-streptococcal glomerulonephritis where anti-C1q autoantibodies were also found to associate with a worse disease course (Kozyro et al., 2008). Why anti-C1q autoantibodies would predominantly enhance the tissue damage in glomeruli and not or less pronounced in other tissues known to contain immune complexes in lupus is currently unknown. The observation that

anti-C1q autoantibodies may specifically target C1q bound to early-apoptotic cells (Bigler et al., 2009) raises the question what the *in vivo* consequences would be of enhanced complement activation on apoptotic cells. One possible scenario could be that the natural mechanisms that would limit excessive complement activation on dying cells would be overruled (Trouw et al., 2007, 2008) resulting in lysis of the cells and exposure of autoantigenic components to the immune system. The observation that anti-C1q autoantibodies are also observed in autoimmune thyroid diseases and that their levels correlate with thyroid function (Potlukova et al., 2008) may suggest that the effect of anti-C1q antibodies amplifying immune-complex mediated damage only in the kidney is incomplete and that the presence of anti-C1q antibodies may enhance tissue damage in several other, unexpected clinical conditions.

In conclusion; anti-C1q autoantibodies play an important role in the clinical management of LN. Testing for anti-C1q autoantibodies in large well defined cohorts of several diseases, preferable in a prospective study design, is likely to provide additional clinical conditions for which the testing for anti-C1q autoantibodies would have clinical implications.

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Monocyte expressed macromolecular C1 and C1q receptors as molecular sensors of danger: implications in SLE

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The ability of circulating blood monocytes to express C1q receptors (cC1qR and gC1qR) as well as to synthesize and secrete the classical pathway proteins C1q, C1r, and C1s and their regulator, C1-INH is very well established. What is intriguing, however, is that, in addition to secretion of the individual C1 proteins monocytes are also able to display macromolecular C1 on their surface in a manner that is stable and functional. The cell surface C1 complex is presumably formed by a Ca^{2+}-dependent association of the $C1r_2{\cdot}C1s_2$ tetramer to C1q, which in turn is anchored via a membrane-binding domain located in the N-terminus of its A-chain as shown previously. Monocytes, which circulate in the blood for 1–3 days before they move into tissues throughout the body, not only serve as precursors of macrophages and dendritic cells (DCs), but also fulfill three main functions in the immune system: phagocytosis, antigen presentation, and cytokine production. Since the globular heads of C1q within the membrane associated C1 are displayed outwardly, we hypothesize that their main function – especially in circulating monocytes – is to recognize and capture circulating immune complexes or pathogen-associated molecular patterns in the blood. This in turn may give crucial signal, which drives the monocytes to migrate into tissues, differentiate into macrophages or DCs, and initiate the process of antigen elimination. Unoccupied C1q on the other hand may serve to keep monocytes in a pre-dendritic phenotype by silencing key molecular players thus ensuring that unwarranted DC-driven immune response does not occur. In this paper, we will discuss the role of monocyte/DC-associated C1q receptors, macromolecular C1 as well as secreted C1q in both innate and acquired immune responses.

Keywords: c1q, DC and C1, monocyte C1, C1q in SLE, C1q in autoimmunity, C1q and C1q receptors

INTRODUCTION

Monocytes serve a critical role in adaptive and innate immunity not only by serving as precursors of macrophages and myeloid dendritic cells (DCs), but also by their function in phagocytosis, antigen processing, and presentation, as well as secretion of pro- and anti-inflammatory cytokines. Monocytes are produced by the bone marrow from hematopoietic stem cell precursors called monoblasts, and circulate in the bloodstream for about 3 days before they migrate into tissues throughout the body where they develop into different types of macrophages and DCs (1, 2). Although their inherent plasticity allows them to develop into various types and subtypes, three major types of blood monocyte subpopulations are recognized: the classical monocyte or CD14++ CD16–, which expresses high level of the LPS receptor (CD14) but no expression of Fc receptor (CD16), and the non-classical monocyte or CD14+ CD16++ characterized by low level expression of CD14 but higher level of CD16 expression. The third is an intermediate between the two, which expresses high level CD14 but low level CD16 (CD14++ CD16+) and is postulated to serve as a transitory link in the maturation process of the classical monocyte into the activated, non-classical monocyte (1, 2). The various monocyte types have been shown to exhibit distinct phenotype and function. Upon activation with microbial antigens, the non-classical monocytes are activated to produce high amounts of pro-inflammatory cytokines including tumor necrosis factor and IL-12 and exhibit higher potency in antigen presentation.

One of the major functions of blood monocytes is to eliminate antibody- or complement-opsonized microbes through either phagocytosis or by binding directly to the pathogen via pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular ligands (3). Emerging among these PRRs are cC1qR and gC1qR, which in addition to their primary ligand, C1q, can also independently recognize a vast array of plasma proteins as well as pathogen-associated molecular ligands (4, 5). Moreover,

Abbreviations: cC1qR, receptor for the collagen-like region of C1q; DC, dendritic cell; gC1qR, receptor for the globular heads of C1q; iDC, immature DC; mDC, mature DC.

monocytes express on their surface an intact macromolecular C1 as well as C1-INH (6, 7) with the globular heads free to recognize antigens. However, despite the available body of evidence showing the presence of macromolecular C1 on the surface of circulating blood monocytes (7), not much is known about its physiologic function. Based on the available data, we postulate that C1q – within the C1 complex expressed on circulating monocytes – may serve not only as a molecular sensor of danger but also as a molecular guarantor of steady state. Thus, while in the steady state, C1q within the C1 complex would regulate early processes that maintain cells in the monocyte or monocyte-like lineage, (*innate immunity*); recognition of "danger" would impart a license that drives monocytes toward the DC lineage (*adaptive immunity*). Deficiency in C1q therefore would disrupt this equilibrium.

DENDRITIC CELLS AND AUTOIMMUNE DISEASES

Dendritic cells are a complex lineage of antigen presenting cells (APCs) that orchestrate a variety of immune responses (3, 8–14). Although B and T cells are known to be the mediators of acquired immunity, their function is under the control of DCs. 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 (9). While B cells, the precursors of antibody-secreting cells, can directly recognize native antigen through their B-cell receptors, T lymphocytes need the antigen to be processed and presented to them by APCs such as DCs. The T cell antigen-receptors (TCRs) recognize fragments of the processed antigens bound to MHC molecules on the surface of DCs (9). The peptide-binding molecules on the APC are of two types: MHC class I (MHC I), which stimulates cytotoxic CD8+ T cells, and MHC class II (MHC II), which stimulates helper CD4+ T cells (8). A second co-stimulatory signal that is critical for T cell activation is the interaction of CD28 on the T cell and CD80/CD86 on the APC such as DC. 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 (11, 14).

Dendritic cell precursors circulate in the bloodstream as monocytes, which are continuously generated from bone marrow progenitors. Migration into non-lymphoid organs induces differentiation of DC precursors into DCs that become resident tissue cells of the interstitium of peripheral organs or skin (9). These tissue-resident DCs are thought to be in an immature state [immature DCs (iDCs)] and are specifically characterized by high phagocytic activity and the ability to capture self and foreign antigens. The presence of specific lectins, such as DC-SIGN, Langerin, and mannose receptors on their surface allows iDCs to recognize invading bacteria or viruses (10, 13). Moreover, the expression of molecules such as $\alpha_v\beta_5$ integrin and CD91 enables them to recognize and engulf self-antigens including those associated with apoptotic cells (10). Therefore, imDCs can interact with self-components, and virtually every antigen present in the periphery can be processed after engulfment and presented as peptide–MHC complexes on the DC surface (8, 11). Fortunately, tolerogenic mechanisms exist to prevent inappropriate autoimmune responses. Presumably, under *steady state* conditions DCs remain immature and start migrating towards lymph nodes upon partial activation signaling. Once they have reached the T cell area, these semi-mature DCs may induce tolerance by numerous mechanisms (3, 11, 14). In contrast, when DCs encounter a peripheral microenvironment characterized by pro-inflammatory factors and antigenic material, massive migration and maturation is triggered by molecules such as LPS, bacterial DNA, and double stranded RNA, which are recognized by specific Toll-like receptors (3, 12). Cytokines such as TNF-α and IL-1β found in the inflammatory compartment are also important participants in the DC maturation/activation process (9). The maturation of DCs is associated with the up-regulation of co-stimulatory molecules such as CD40, CD80, CD86, and CD58, secretion of cytokines, such as TNF-α, IL-6, and IL-12p70, the loss of endocytic phagocytic receptors, high levels of MHC I, II, CD83, and acquisition of high cellular mobility (13). The mature DCs migrate to the T cell areas of local lymph nodes where they are retained via specific chemokine interactions involving CCR7. Subsequent screening events result in engagement of the appropriately matched T cell-MHCI/MHCII receptors. Priming of naïve CD4+ T cells takes place when DCs engage CD40L. Because of their unique ability to initiate immune responses against invading pathogens as well as against peptides derived from self-proteins, DCs play an important role in the development of autoimmune diseases. However, a variety of mechanisms impact on the immunogenicity of DC in order to prevent autoimmune responses including *local factors* that facilitate *decisions* about the nature and subset of T cell response (15–18). The various signals that influence the DC are not yet fully elucidated, but these signals are likely to depend on the type and dose of antigen, the microenvironment of the DC–antigen encounter, the number, subset, and phenotype of the DC involved, the microenvironment of the secondary lymphoid organs where the antigen is presented, and finally the *local synthesis* of modulatory proteins including *complement proteins*, such as C1q, and its C1q receptors each of which is capable of recognizing and capturing self or non-self antigens.

EXPRESSION OF C1 AND C1q RECEPTORS ON MONOCYTES AND DENDRITIC CELLS

The synthesis of the subunits of the C1 complex (C1q, C1s, C1r), and its regulator C1 inhibitor (C1-INH) by human monocytes has been shown independently by several investigators (6, 19–24). However, the expression of the *intact* macromolecular C1 on the *cell surface* of monocytes and DCs has been described only recently (7). The first indication of the existence of a membrane form of C1 was intimated by the finding that monocyte-derived macrophages were able to synthesize and express a membrane anchored form of C1q (25). Furthermore, these original experiments showed that: (i) the surface expressed C1q was tightly and irreversibly anchored into the cell surface membrane with its globular heads displayed outwardly as evidenced by the fact that it was able to bind Fc, polyanions, the lipid A part of LPS, Gram-negative bacteria such as *S. minnesota*, as well as porins (outer membrane bacterial proteins); (ii) the A-chain of C1q contains amino acid sequences with properties characteristic of an integral type II membrane protein; and (iii) the surface molecule could only be liberated by detergent or repeated freeze-thawing (26, 27). However, these observations presumed that macrophages and *not their* monocyte precursors

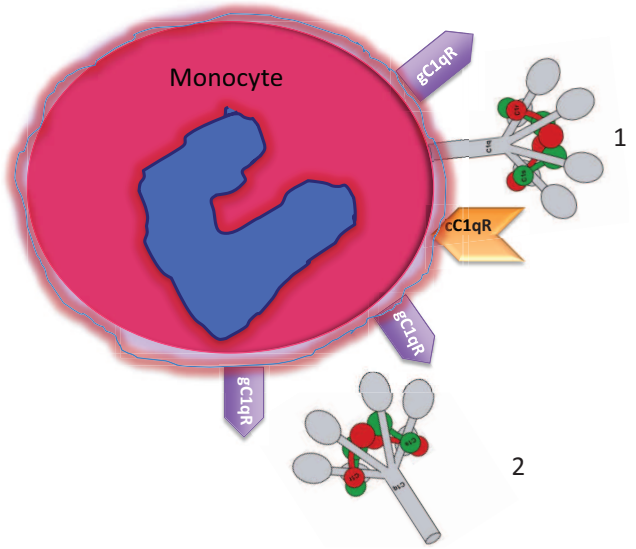

FIGURE 1 | Circulating blood monocytes express C1 and C1qRs. (1) Membrane anchored C1q displays its globular heads free to interact with antigens or immune complexes. (2) Some of the membrane anchored C1q is cleaved off and secreted into the pericellular milieu and is able to bind to the cell surface via gC1qR. The C1q figure was obtained from Gérard Arlaud.

were able to express C1q on their surface. Using more sensitive assays and antibodies, recent experiments from our laboratory have shown that non-stimulated circulating blood monocytes are also able to both synthesize and express all of the components of C1 (C1q, C1r, C1s) as well as C1-INH (7). The C1 complex is presumably assembled around the C1q molecule in a manner that is similar to the C1 complex in plasma, with the C1q molecule serving as an anchored backbone to which the $C1r_2{\cdot}C1s_2$ tetramer is linked via Ca^{2+} ions. Such a configuration, which is similar to the natural configuration of plasma C1 (**Figure 1**) would allow the exposure of the globular heads for immune complex binding or antigen recognition and complement activation. This assumption is also corroborated by the finding that the U937 expressed C1, is able to activate complement (7), and this activation is closely controlled by C1-INH, present on or near the cell surface (6). Collectively, the data suggest that the surface-associated C1 complex can initiate complement activation in response to an extracellular Ag and thus may represent the earliest response to either pathogen-associated or modified self-associated danger signals in blood.

The expression of C1q on monocytes is dependent on the maturation stage (**Figure 2**). While activated monocytes (macrophages) and immature DCs express elevated levels of C1q, this ability is lost when iDCs transition into mature DCs (mDCs) (28, 29). Interestingly, this expression profile is also mimicked by both C1q receptors (cC1qR and gC1qR), which in turn are co-localized with C1q and DC-SIGN on iDCs suggesting that C1q/gC1qR may regulate DC differentiation and function through the DC-SIGN-mediated induction of cell-signaling pathways (30). While inflammatory cytokines and LPS, which induce maturation of DCs, downregulate surface expression of both C1qR molecules, cytokines, and drugs such as IL-10, TNFα, or dexamethasone, that keep DCs phenotypically and functionally immature significantly upregulate the expression of both C1qRs (28).

The significance of C1q in the development of autoimmune diseases such as systemic lupus erythematosus (SLE) has been known for many years (31–33), and its potential role in tolerance induction has been proposed (34, 35). However, neither the mechanism by which C1q determines the activation thresholds of B and T cells nor how C1q deficiency causes incomplete maintenance of peripheral tolerance is clearly understood. The fact that C1q is co-localized with a number of surface molecules such as DC-SIGN, gC1qR, and cC1qR-implies that these molecules may collaborate in antigen recognition and processing (30).

C1 DEFICIENCY AND SLE

Systemic lupus erythematosus – a prototype of a systemic autoimmune disease – affects close to three-quarters of a million individuals in the US and a much higher number worldwide with a frequency that varies by race and ethnicity with higher rates reported among Black and Hispanic people (36). Although it is a multifactorial disease, there is an overwhelming clinical evidence showing that homozygous deficiency in any of the classical pathway proteins – C1q, C1r, C1s, C4, and C2 – predisposes an individual to develop SLE and other autoimmune diseases such as rheumatoid arthritis (RA) (31, 33, 37). Among these proteins, C1q takes a prominent stage in significance as homozygous deficiency or hereditary deficiency due to mutation in the C1q gene is regarded as a strong susceptibility factor for the development of SLE (31, 33). The majority (≥95%) of the known individuals with C1q deficiency are known to have developed clinical syndromes closely related to SLE (31, 33, 38, 39). Interestingly, the majority of the circulating antibodies in SLE are against "modified self" or intracellular proteins (nuclear or cytoplasmic), suggesting that C1q may play an important role in the regulation and processing of these antigens.

There has been an exponential increase in recent years exploring the possibility that C1q is a major link between *innate and acquired immunity*. Some of these studies have been made possible in part due to the availability of $C1q^{-/-}$ animal models (40). As an archetypal pattern-recognition molecule with the ability to sense a wide variety of targets, C1q can engage a broad range of ligands – ranging from pathogen-associated molecular ligands (non-self) to damage-associated molecular targets (altered self) – including "eat me" signals such as self DNA and phosphatidylserine, which are the first structures exposed at the apoptotic cell surface (41–45). These interactions are able to trigger a multiplicity of immunologic functions, which by and large are beneficial to the host. It is not therefore surprising that deficiency in C1q leads to various diseases including otitis media, meningitis, pneumonia (31, 34, 37) as well as autoimmune diseases, such as SLE (31, 46, 47). Several reports have shown that C1q can bind via its globular "heads" to the surface of apoptotic cells (44, 47); and the common autoantigens targeted in SLE are found in high concentrations on the surface of apoptotic cells (44, 47). However, although it is clear that C1q and its receptors play a role in removal of self-waste (44, 47–50) the existence of functionally redundant pathways for apoptotic body clearance gives credence to the postulate that autoimmunity arising from diminished C1q activity could reflect another role of

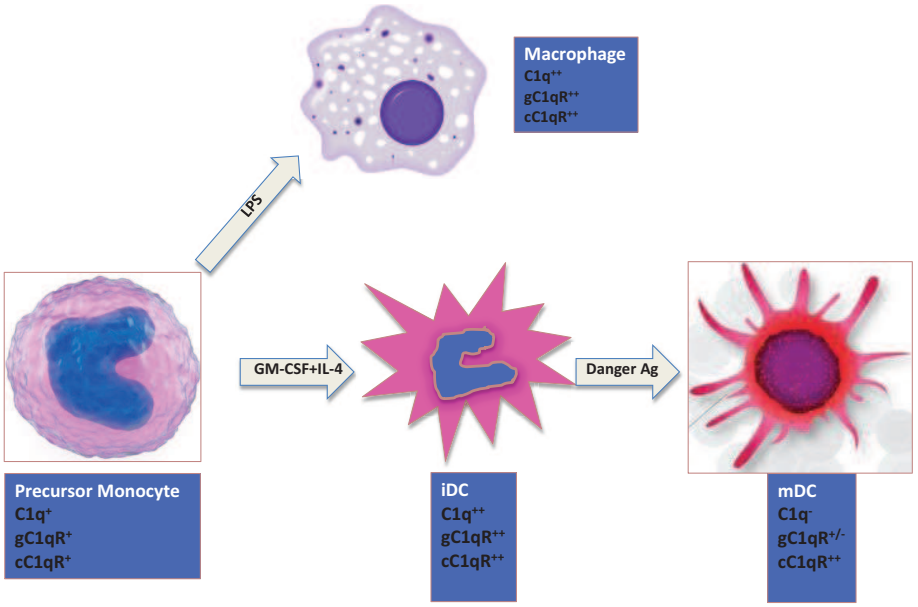

FIGURE 2 | Expression of C1q and C1qRs is maturation dependent. Circulating monocytes express both C1 and C1qRs, which increase during the monocyte to iDC transition in the presence of self- or foreign-danger signals. Fully mature DCs, however, express little or no C1q, low gC1qR but high cC1qR.

C1q in maintaining tolerance (34). Thus, while C1q could provide active protection from autoimmunity by silencing key molecular markers or regulating autoreactive immune cells, its absence or defective expression could lead to a loss of peripheral tolerance as a cumulative result of impaired apoptotic cell clearance in conjunction with negative signaling. For example, incubation of C1q with T cells has been shown to inhibit T cell proliferation presumably by binding to gC1qR on the T cell surface (51) since this activity is mimicked by incubation of cells with mAb 60.11, which recognizes the C1q binding site on gC1qR. Furthermore, although its role is not precisely known, soluble gC1qR is able to bind CD4 thereby blocking HIV-1 viral entry into T cells (52). More importantly, because of its heterotrimeric nature and oligomeric structure – with two distinct structural and functional domains (gC1q and cC1q) – C1q is also able to interact with cell surface receptors via either its cC1q or gC1q domains. Similar to C1q, the C1q receptors are differentially expressed as monocytes go through the maturation process to become mDCs (35). When monocytes are cultured in the presence or absence of GM-CSF + IL-4, nearly all monocyte-derived DCs expressed gC1qR on day 0 (**Figure 3B**), while cC1qR was more variable within the population (**Figure 3A**), despite the consistently elevated expression of its putative surface partner, CD91 (**Figure 3C**). Even though there was a modest reduction in $gC1qR^{+}$ cells by day 4 (**Figure 3B**), the percent of cC1qR expression increased compared to day 0 (**Figure 3A**). Furthermore, mean fluorescence index (MFI) analysis revealed that the amount of cC1qR was dramatically amplified after day 2 (**Figure 3D**), whereas the amount of gC1qR remained at relatively steady levels (**Figure 3E**). 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 in turn 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 (**Figures 3C,F**). Taken together, these data suggest that the regulatory effects of C1q on DC differentiation and function may depend on specific C1q/C1qR interactions; and these interactions may in turn control the transition from the monocyte state (*innate immunity*) toward the professional APC state (*adaptive immunity*). The observation that soluble C1q functions as a "*molecular switch*" during the narrow window – i.e., at the precise period (~day 3) corresponding to firm commitment to the DC lineage – of monocyte to DC transition (35) not only explains why C1q is primarily synthesized, expressed, and secreted by potent APCs, but also why its absence can impair antigen uptake and tolerance.

On the basis of the available data and our own recent findings (35), we speculate that soluble C1q induces inflammatory responses by binding through its gC1q to pathogen-associated molecular patterns (PAMPs) and to modified self antigens (DAMPs), and stimulates phagocytic cells through interactions of its cC1q domain with cC1qR. According to this *hypothesis*, a normal response to "*danger*" would involve up-regulation of cC1qR on iDC to ensure uptake of noxious agents utilizing the Ag-retrieving functions of C1q (44, 48). In the context of inflammatory stimuli, DC maturation would ensue allowing adaptive immune responses against the initiating agent. In contrast, C1q that is free of antigenic cargo (i.e., in the absence of *danger*) does not support full commitment to the DC lineage, and instead keeps them in a monocytic phenotype as represented during steady state.

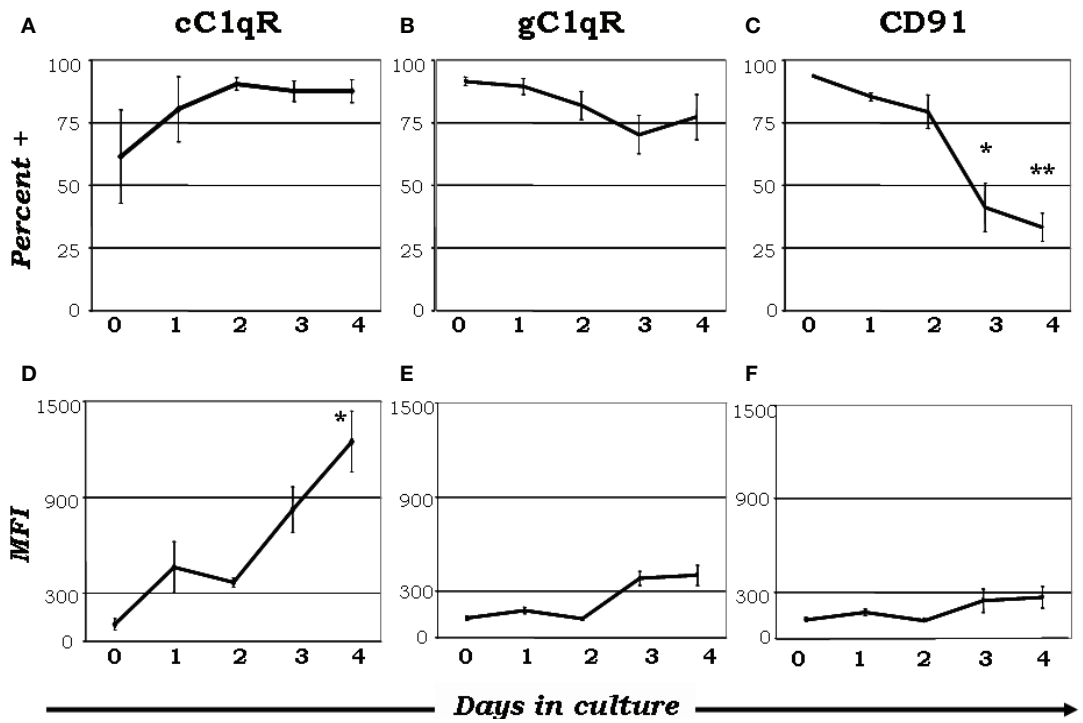

FIGURE 3 | Maturation dependent expression of C1qRs. C1q receptors show varied expression on mono-DC precursors. Mononuclear cells were cultured in the presence of GM-CSF + IL-4, and collected and analyzed on days 0–4 for the expression of cC1qR **(A,D)**, gC1qR **(B,E)**, and CD91 **(C,F)** for both 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. Experiments were gated on DR+ cells, $*p < 0.05$, $**p < 0.01$ ($n \geq 4$) [adapted from Ref. (35)].

Therefore, 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 (**Figure 4**).

Similar to the suppressive effect of C1q on T cells acting through gC1qR/C1q interactions (51, 53), we hypothesize that the regulatory effects of C1q on monocyte/DC precursors may occur via engagement of gC1q. 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 or infection or inflammation). The dichotomy of the two apparently opposing roles of C1q in turn are a result of the binding orientation of C1q – *heads* versus *tails* – and the specific receptors engaged (*gC1qR* versus *cC1qR*) on the cell surface. Such duality of function would be very similar to the role of surfactant proteins (Sp)-A and Sp-D in the lung, which help maintain the steady state environment via binding to the ITIM-containing SIRPα through their globular head domains or initiate ingestion and pro-inflammatory responses through the collagenous tails and cC1qR (calreticulin)/CD91 (54). Furthermore, although the significance of these motifs has yet to be elucidated, another intriguing observation in this context is the presence of dueling ITAM/ITIM motifs around the tyrosines at positions 224 and 236, respectively on gC1qR, which is unique for a molecule that is both an intracellular protein and a cell surface receptor (55). The observations described above together with the finding that there is a clear differential pattern of C1qR expression during the monocyte to DC transition (28, 35), allows us to hypothesize that preferential engagement of distinct regions of C1q (globular heads versus collagen tails) takes place during different stages of DC growth. Accordingly, the globular head domain of the molecule would mediate regulatory effects of C1q. Since the collagen tail of the C1q in plasma or on the cell surface would be occupied, this association only allows the C1q subunit to bind gC1qR and not cC1qR on the cell surface. In support, we have found that some or all of the C1q detected on the monocyte surface is part of the C1 complex (7). Furthermore, cC1qR has been shown to bind C1q only when it undergoes a conformational change (e.g., through immobilization on a surface) (56). Such altered conformation may take place when C1q binds antigen or immune complexes.

EFFECT OF C1q ON DC DIFFERENTIATION AND FUNCTION

The development of monocyte-derived DCs from human peripheral blood (PB) is marked by the rapid loss of CD14 (receptor for LPS), up-regulation of CD11c [integrin, α_x (complement component 3 receptor 4 subunit (CR4)], HLA-DR (MHC class II), and co-stimulatory and maturation associated molecules (such as CD86, CD80, CD83) (57–59). Distinct subsets of DC precursor

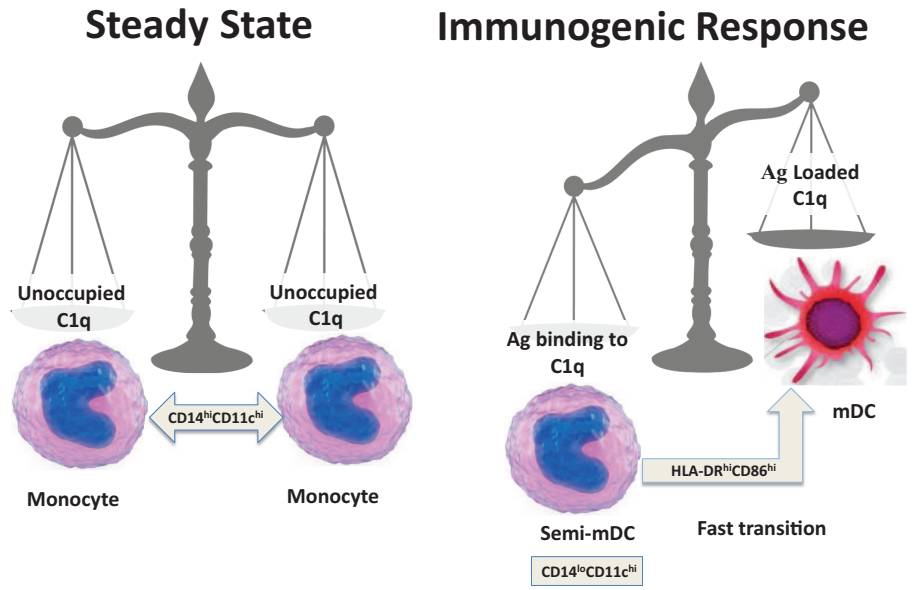

FIGURE 4 | Schematic representation of a fine-tuned balancing act. The transition from steady state to immunogenic response depends on the encounter between an antigen and C1q.

populations have been described to arise from human PB monocytes treated with GM-CSF + IL-4. These include $CD14^{-}CD11c^{+}$, $CD14^{-}CD11c^{-}$, and $CD16^{\pm}CD14^{+}CD11c^{+}$ cells (58, 59). While the majority of iDCs avidly ingest potentially antigenic material as a prerequisite for an immunogenic response, failure to undergo terminal maturation into APCs results in a potentially tolerogenic state (8, 11). Targeting of various uptake receptors, notably of C-type lectin receptors (e.g. DC-SIGN, Langerin, BDCA-2, Dectin-2, etc.) by self-derived Ag on iDCs leads to tolerance by default (8, 11), whereas pathogen-derived Ag might simultaneously signal through TLRs and C-type lectins, thus induce immunity (8, 11).

Although somewhat contradictory to the findings of others, which showed that C1q induces maturation of DCs (60), recent data from our laboratory (35) show that: (i) even in the presence of DC growth factors, exogenously added C1q is able to inhibit the expression of monocyte-derived DC maturation markers such as CD86, a required co-stimulatory molecule for T cell activation (**Figures 5A,B**); (ii) C1q promotes the development of distinct iDC subsets $CD14^{hi}CD11c^{hi}CD16^{\pm}$HLA-$DR^{hi}CD86^{dim}$ (**Figures 5C,D**) compared to $CD14^{-}CD11c^{hi}CD16^{-}$HLA-$DR^{hi}CD86^{hi}$ cells, which develop without C1q. The decreased expression of maturation dependent markers on C1q treated versus untreated cells substantiates the notion that C1q alters monocyte to DC differentiation. In addition to these observations, our previously published results (7, 35) indicate that Ag-free, extracellular C1q exerts a regulatory signaling function through engagement of the globular heads by surface gC1qR and thus modulate the differentiation of monocyte-DC precursors and induce the development of $CD14^{hi}CD11c^{hi}CD16^{+/-}$ cells. Therefore, the decreased expression of DC maturation dependent markers and the increased expression of monocyte associated surface molecules in the presence of C1q support the concept that the developing cells acquire features of both monocyte and DC. Because C1q is structurally similar to SP-A and SP-D, it might also perform a *dual* function in that it may help maintain the steady state environment via binding to ITIM-containing surface molecules on cells (54, 61) including gC1qR through its globular head domains. Accordingly, occupancy of the gC1q by foreign Ag would induce a conformational change (56) thereby making the collagen tail available for binding to cC1qR. The ensuing cell differentiation and signaling events would thus support rapid DC maturation and immune activation. Conversely, self-derived Ag bound C1q might relay signals through C1q or C-type lectin receptors only, thus inducing a strong tolerogenic response, resulting in development of tolerogenic DC and Treg activation.

INSIGHTS IN TO THE MECHANISMS OF C1q-MEDIATED MONOCYTE/DC SIGNALING

To date, the information available on the signaling events regulated by C1q in DCs is very limited. Some studies using murine bone marrow-derived DCs, have shown that C1q treatment suppresses IL-12 production, and reduces the phosphorylation of p38, ERK1/2, c-Jun N-terminal kinase, and extracellular signal-regulated kinase after stimulating the cells with LPS, CpG oligodeoxynucleotides, or anti-CD40 antibodies (62, 63). This is contrary to the findings by others that C1q induces maturation of DCs and secretion of IL-12 and TNF-α and elevated their T cell stimulating capacity (60). However, a definitive mechanism that mediates C1q signaling in human DCs has not been described. Understanding the molecular mechanisms of how C1q regulates adaptive immune functions via iDCs in the absence of infection or inflammation is therefore highly significant. Since our data indicate that CD14 levels are markedly increased on

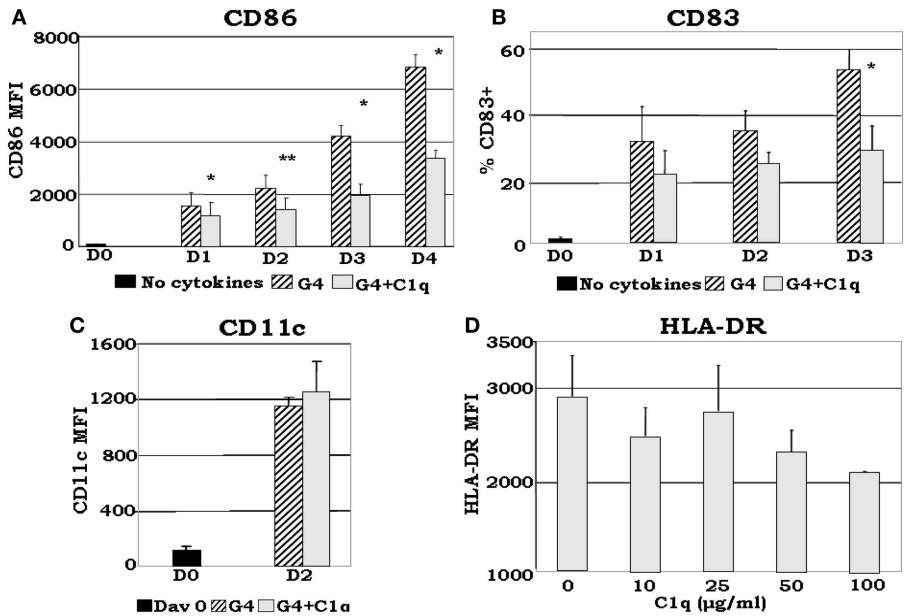

FIGURE 5 | C1q delays GM-CSF + IL-4 induced DC maturation. Monocyte-DCs were isolated and cultured in the presence of GM-CSF + IL-4 (G4) and with or without 25 μg/ml C1q **(A–C)**. For the dose response experiments several concentrations of C1q were added as indicated **(D)**. Cells were collected on 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 DR+ cells for all experiments [adapted from Ref. (35)].

mono-DCs when they are cultured in the presence of C1q (35), signaling blockades that may potentially be mediated by CD14 should be evaluated. LPS triggers CD14/TLR4 mediated signaling to induce cell differentiation via TRAF6 and IRAK4 signaling. Although the effect of C1q on $CD14^-$ and TLR-mediated signaling cascades in mono-DCs has not been yet fully investigated, ligand engagement of gC1qR (HCV core protein and mAb) has been shown to increase PI3K activation and Akt phosphorylation in LPS stimulated monocytes in an ERK independent manner (64). Moreover, DC-SIGN, which forms a molecular complex with C1q and gC1qR on the surface of iDCs (30), has been shown to increase phosphorylation of Raf-1 on Ser338 and Tyr340/341 in a ligand-dependent manner (65). Furthermore, stimulation of DC-SIGN with the mannose receptor-1 (MR-1) Ab has been shown to induce activation of the MEK/ERK kinase cascade (66). Whether direct stimulation of C1q participates in these signaling pathways, however, still remains to be investigated.

CONCLUDING REMARKS AND FUTURE OUTLOOK

The present paper is intended to provoke a debate and inject novel insights into the endlessly intriguing question: *Why does homozygous deficiency in C1q predispose an individual to develop SLE and other autoimmune diseases?* Although the contribution of C1q to the process of apoptotic cell clearance is unquestionable – and is supported by robust experimental data – the presence of functionally redundant pathways for removal of apoptotic bodies together with the direct effect of C1q on monocytes/DCs and T cells would suggest that, in addition to its role in apoptotic clearance, locally secreted C1q – which is DC maturation dependent – determines the activation thresholds of B and T cells and that C1q deficiency causes incomplete maintenance of peripheral tolerance. Therefore, while in the steady state, C1q would regulate cells during the early DC differentiation events by silencing critical phagocytic or stimulatory markers requisite for antigen presentation (*innate immunity*), recognition of "danger" would, on the other hand, impart a signal that drives monocytes toward the DC lineage (*adaptive immunity*). Deficiency in C1q therefore will disrupt this equilibrium. In addition, 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. The distinct binding orientation of C1q (heads versus tail) on the monocytes and iDCs also suggests that specific C1q/C1qR interactions – and possibly other surface molecules – may regulate cells as they transition from the monocyte state toward the professional APC state. The presence of C1q together with C1r, C1s, and C1-INH on the monocyte surface with its versatile

antigen-capturing region displayed outwardly also supports the concept that monocytes may represent the first sentinels of danger in the blood.

However, although these postulates are conceptually appealing and are supported by experimental data, future studies, which focus on the role of C1qRs, C1q, and C1 within the microenvironment of the monocyte/DC–antigen encounter will be necessary if we are to gain insight into why deficiency in any of the components of C1 leads to the development of autoimmunity. More importantly, information derived from such studies will not only provide new groundwork for future undertakings related to innate and acquired immunity, but also would give credence to targeted modulation of C1/C1q-mediated DC function as new treatment option aiming at alleviating DC-driven autoimmune responses.

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Peptide inhibitor of complement C1, a novel suppressor of classical pathway activation: mechanistic studies and clinical potential

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The classical pathway of complement plays multiple physiological roles including modulating immunological effectors initiated by adaptive immune responses and an essential homeostatic role in the clearance of damaged self-antigens. However, dysregulated classical pathway activation is associated with antibody-initiated, inflammatory diseases processes like cold agglutinin disease, acute intravascular hemolytic transfusion reaction (AIHTR), and acute/hyperacute transplantation rejection. To date, only one putative classical pathway inhibitor, C1 esterase inhibitor (C1-INH), is currently commercially available and its only approved indication is for replacement treatment in hereditary angioedema, which is predominantly a kinin pathway disease. Given the variety of disease conditions in which the classical pathway is implicated, development of therapeutics that specifically inhibits complement initiation represents a major unmet medical need. Our laboratory has identified a peptide that specifically inhibits the classical and lectin pathways of complement. *In vitro* studies have demonstrated that these peptide inhibitors of complement C1 (PIC1) bind to the collagen-like region of the initiator molecule of the classical pathway, C1q. PIC1 binding to C1q blocks activation of the associated serine proteases (C1s–C1r–C1r–C1s) and subsequent downstream complement activation. Rational design optimization of PIC1 has resulted in the generation of a highly potent derivative of 15 amino acids. PIC1 inhibits classical pathway mediated complement activation in ABO incompatibility *in vitro* and inhibiting classical pathway activation *in vivo* in rats. This review will focus on the pre-clinical development of PIC1 and discuss its potential as a therapeutic in antibody-mediated classical pathway disease, specifically AIHTR.

Keywords: complement, classical pathway, C1q, MBL, peptide, inhibitor, ABO incompatibility, AIHTR

INTRODUCTION

Activation of the classical pathway of complement is mediated via C1, a multimolecular complex composed of the recognition molecule C1q and the associated serine proteases C1r and C1s (1). IgM or clustered IgG serves as the principal ligand for C1q, resulting in the sequential activation of C4 and C2 to form the classical pathway C3-convertase leading to C3 and C5 activation. This cascading activation sequence generates a number of inflammatory effector molecules designed to limit infection by invading pathogens. Additionally, C1 plays an essential housekeeping role by recognizing and clearing cellular debris, immune complexes, altered self, and apoptotic cells (2) as well as identifying abnormal structures such as beta-amyloid fibrils (3, 4) and the pathological form of the prion protein (5, 6). The ability of this molecule to distinguish self from non-self is critical for immune tolerance and homeostasis (7).

Under normal circumstances, complement activation is tightly controlled by a number of regulatory proteins to minimize host tissue damage. However, aberrant activation of the classical, lectin, or alternative pathways of complement leads to significant host tissue damage in many inflammatory diseases (8). In the case of the classical pathway of complement, irregular activation of this pathway has been implicated in cold agglutinin disease (CAD) (9), acute/hyperacute transplantation rejection (10), and other inflammatory and autoimmune conditions (11). In the field of hematology, inappropriate activation or dysregulated control of complement activation plays a central role in hemolytic disease processes such as acute intravascular hemolytic transfusion reaction (AIHTR) (12), autoimmune hemolytic anemia (13), and hemolytic disease of the newborn (14). In these disease processes, preexisting antibodies, typically natural antibodies, bind to erythrocyte surface antigens initiating classical complement pathway activation via C1 (15). Subsequent terminal complement cascade activation leads to membrane attack complex (MAC) formation on the erythrocyte surface and intravascular hemolysis. In the case of an AIHTR (such as an ABO incompatible transfusion), recipient serum containing antibody that specifically recognizes antigens on the surface of the donor's red blood cells (RBCs)

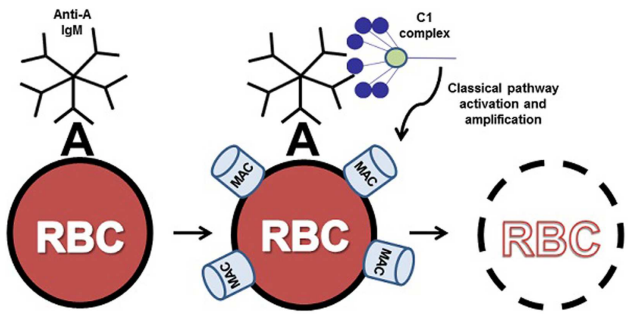

FIGURE 1 | ABO incompatible destruction of RBCs by the classical pathway of complement. In this example, transfused cells expressing A antigen have been exposed to incompatible recipient serum containing anti-A IgM. The bound IgM molecule is then recognized by serum complement C1 complex. Upon binding to IgM, C1 activates the classical pathway, resulting in complement deposition on the donor cell and eventual clearance or lysis of host RBCs by the membrane attack complex (MAC). For clarity, only C1 binding and MAC formation are illustrated here.

results in classical pathway mediated rapid intravascular hemolysis of donor RBCs within minutes (**Figure 1**). Due to the rapidly amplifying cascade driving this process, AIHTR frequently results in shock, disseminated intravascular coagulation, acute renal failure due to hemoglobinuria, and death (16). To date, there are no pharmacological interventions to treat the underlying mechanisms of this disease processes such that treatment is primarily supportive (17, 18). Thus, there is a critical need for inhibitors of the classical pathway that can block complement activation at the level of C1 and prevent the avalanche of complement activation at the initiation event of the cascade. In this review, we describe the identification and characterization of a novel inhibitor of C1 termed peptide inhibitor of complement C1 (PIC1) and discuss its potential as an anti-complement therapeutic for transfusion medicine applications.

DISCOVERY AND CHARACTERIZATION OF PIC1

The findings that led to the discovery of the complement inhibitory peptide PIC1 in our laboratory initially came from our research program on human astrovirus capsid assembly. Below, we discuss how PIC1 has evolved from a capsid protein of 787 amino acids to a 15 amino acid peptide that is unique in nature.

HUMAN ASTROVIRUS COAT PROTEIN INHIBITION OF THE CLASSICAL AND LECTIN PATHWAYS

Human astroviruses (HAstVs) are a major cause of diarrhea in human infants (19). The virus replicates in intestinal epithelial cells, but produces no inflammation in the intestinal tissues (20). We showed in hemolytic complement assays that lysates made from cell culture infected with HAstV serotypes 1, 2, 3, and 4 all strongly inhibited serum complement activation, >84% (21). Human astrovirus type 1 (HAstV-1) is the most common serotype worldwide (22). Recombinantly expressed HAstV-1 coat protein (CP) was able to inhibit complement-mediated hemolysis >90%. Subsequent testing showed that CP was able to inhibit the activation of C3, C5, and C5b-9 in serum, demonstrating that the generation of downstream effectors was inhibited (21). CP strongly inhibited activation of the classical pathway and antibody-initiated complement activation in a variety of systems, but had no significant effects on the alternative pathway. CP inhibited activation of C1, as assayed by C1s activation, preventing the activation of C4 in serum. Moreover, complement activation could be restored with the addition of exogenous C1. CP bound to both the globular head regions and collagen-like regions (CLRs) of C1q, but had higher affinity for the CLR, where the C1s–C1r–C1r–C1s tetramer is nestled. C1s was displaced from intact C1 by CP, suggesting the displacement of the cognate serine protease(s) is the likely mechanism of inhibiting C1 activation (23). CP also inhibited mannan activation of MBL/MASP2 in human serum (23). MBL with a point mutation that prevents binding to MASP2 (i.e., Lys55Gln substitution) cannot bind CP, suggesting that CP competes for the cognate serine protease binding site of MBL, similar to C1 (23).

PIC1 INHIBITION OF ANTIBODY-INITIATED COMPLEMENT ACTIVATION

Within the amino acid sequence of the CP molecule, we identified a 60-residue region of homology corresponding to human neutrophil peptide 1 (HNP-1) (**Figure 2**). HNP-1 has been identified by other investigators as an inhibitor of C1 and MBL (24, 25). From the region of homology found within the CP molecule, two synthetic peptides were generated of 30 residues each (**Figure 2**). The PIC1 derivative coat protein peptide 1 (CPP1) inhibited the binding of CP to C1q and inhibited C1 activation, as assayed by C1s cleavage, suggesting a similar mechanism of action as the parent compound (**Table 1**) (26). Coat protein peptide 2 did not demonstrate either of these properties, indicating that the active region of CP could be successfully narrowed to a small peptide construct. A further derivative of CPP1 with an alanine substitution at position 23 (E 23A), as indicated by bold red in **Table 1**, demonstrated superior inhibitory activity to CPP1 (26). Additional truncations demonstrated that removing the central region of CPP1 (Δ8–22) yielded a functional peptide of 15 residues (**Table 1**) (26). The Δ8–22 construct inhibited antibody-initiated complement activation to a greater degree than CPP1, as demonstrated in hemolytic as well as C4 activation assays and similar to the parent CP. As a smaller peptide with similar potency to the E23A construct, Δ8–22 still had minimal effect on the alternative pathway (26).

FURTHER OPTIMIZATION OF PIC1 DERIVATIVES

In an attempt to further optimize the PIC1 Δ8–22 derivative and improve its solubility properties, the peptide sequence was rearranged such that most of the hydrophobic residues were placed at the N terminus and the hydrophilic residues were placed at the C terminus. The rearranged PIC1 derivative was termed polar assortant (PA) (**Table 1**) and demonstrated superior complement inhibitory activity to all other derivatives previously reported (27). A deletion analysis of PA showed that loss of one amino acid residue from the N or C terminus destroyed inhibitory activity suggesting that 15 residues may be the optimal length of this PIC1 derivative (27). An alanine scan and a scan substituting each residue of PA with a negative or positively charged amino acid (40 total substitutions analyzed) revealed that PA was the most potent PIC1 analog capable of inhibiting classical pathway mediated complement activation [Ref. (27), and data not shown]. PA was also demonstrated to specifically bind to the CLR of C1q by ELISA and

```
CP        79 PAICQRATATLGTVGSNTSGTTEIEACILLNPVLVKDATGSTQFGPVQALGAQYSMWKLKY 139
HNP-1      0 ACYCR-----------------IPACI----------AGERRYGTCIYQG---RLWAFCC 30
               .. *:                * ***          :*. ::*.    *    :* :

CPP1         PAICQRATATLGTVGSNTSGTTEIEACILL
CPP2                                 NPVLVKDATGSTQFGPVQALGAQYSMWKLKY
```

FIGURE 2 | Alignment of CP with the HNP-1 molecule using ClustalW analysis. Homologous residues are identified as follows: identical (*), conserved (:), and semi-conserved (.) residues. CPP1 and CPP2 were synthesized based upon this alignment.

Table 1 | PIC1 derivatives.

Peptide	Peptide sequence (N → C)	Classical pathway inhibition values using 0.77 mM peptide (%)
CPP1	PAICQRATATLGTVGSNTSGTTEIEACILL	50
E23A	PAICQRATATLGTVGSNTSGTT**A**IEACILL	80
Δ8–22	PAICQRAEIEACILL	70
PA	IALILEPICCQERAA	85

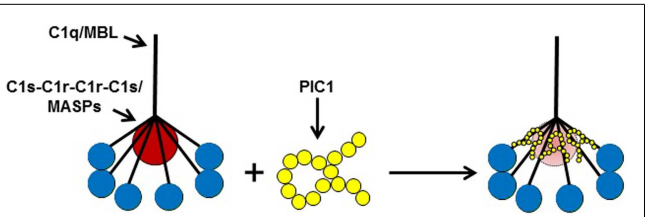

FIGURE 3 | A model of PIC1 interfering with the normal association of C1q/MBL with their cognate serine proteases (C1s–C1r–C1r–C1s/MASPs). Our data suggest PIC1 functionally disrupts the C1s–C1r–C1r–C1s/MASPs interaction with collagen-like region (CLR) of C1q/MBL, respectively. By virtue of PIC1 binding to this region of the CLR, the serine proteases are hypothesized to be in suboptimal conformation for autoactivation and initiation of the classical and/or lectin pathways of complement.

bound to purified C1q with a mean equilibrium dissociation constant (K_D) of 33.3 nM as determined by surface plasmon resonance (27). It has been previously demonstrated by surface plasmon resonance that immobilized C1q binds to its cognate serine proteases (C1s–C1r–C1r–C1s) with a K_D of 2.72 nM (28). The binding of PA to C1q with similar affinity for C1s–C1r–C1r–C1s (i.e., nM range) is consistent with our hypothesis that the PIC1 peptides may function by displacing, at least partially, C1s–C1r–C1r–C1s from the CLR of C1q thereby changing the optimal conformation of the serine protease tetramer and preventing autoactivation. A hypothetical model of the mechanism by which PIC1 derivatives inhibit C1 and MBL activation is presented in **Figure 3**.

The PIC1 PA derivative, to our knowledge, is unique in nature (**Figures 4A,B**) as it does not possess any identifiable similarity with known proteins by NCBI blast search. It is 15 residues in length with a pI of 4.53 and a molecular weight of 1643 Da. While our data to date suggest that PA disrupts the interaction of C1s–C1r–C1r–C1s and MASP2 with the CLR of C1q and MBL, respectively, the precise molecular details of this interaction are unclear. As mentioned above, astrovirus CP does not bind an MBL mutant in which the lysine residue that are critical for MASP2 binding to the CLR of MBL has been substituted with another residue (23). This suggests that PIC1 derivatives may bind to the same critical lysine residue on the MBL and C1q molecules. While it is obvious to suggest that this reactive lysine residue may interact with either of the negatively charged glutamic acid residue on PA at positions 6 and 12 (**Figures 4A,B**), substitution of either of these residues individually or together with alanine had no detrimental effect on PA inhibitory activity (27), suggesting that binding of these derivatives to C1q was not compromised. It is highly conceivable that PIC1 may bind to the C1q/MBL CLR through other interactions such as hydrophobic and hydrogen bonding. Due to the large number of hydroxylated residues that exist in the CLR of C1q and MBL, extensive hydrogen bonding with PIC1 is an entirely feasible mechanism by which PIC1 may bind these molecules. Studies of PIC1 interaction with C1q using protein footprinting techniques combined with mass spectrometry are currently underway in our laboratory. The recent development of a system to produce the A, B, and C chains of C1q recombinantly (29) also provides a means to produce specific mutations in the C1q molecule, which would provide an invaluable resource in mapping the regions of interaction between C1q and PIC1. Precise details on the interaction of PIC1 with C1q and MBL would also benefit from structural studies utilizing X-ray crystallography and/or nuclear magnetic resonance imaging to directly visualize structural interactions. A hypothetical model of PIC1 PA is shown in **Figure 4C**.

PRE-CLINICAL DEVELOPMENT OF PIC1

PIC1 INHIBITION OF ABO INCOMPATIBILITY *IN VITRO* AND *IN VIVO* INHIBITION OF COMPLEMENT

To evaluate the ability of PIC1 derivatives to inhibit ABO mediated RBC lysis, two PIC1 analogs were tested in an *in vitro* model of ABO incompatibility. E23A and an acetylated version of PA were both demonstrated to dose-dependently inhibit lysis of human AB RBCs incubated with human O serum in a modified hemolytic assay (27). Acetylated PA has identical inhibitory activity compared to unmodified PA (27). To preliminarily assess the *in vivo* complement suppression profile of these two derivatives, 20 mg of E23A and an acetylated version of PA were injected into 250 g male Wistar rats. Both peptides were able to cross the species barrier and inhibit serum complement activity in these animals as assessed by hemolytic assay using serum purified from the blood

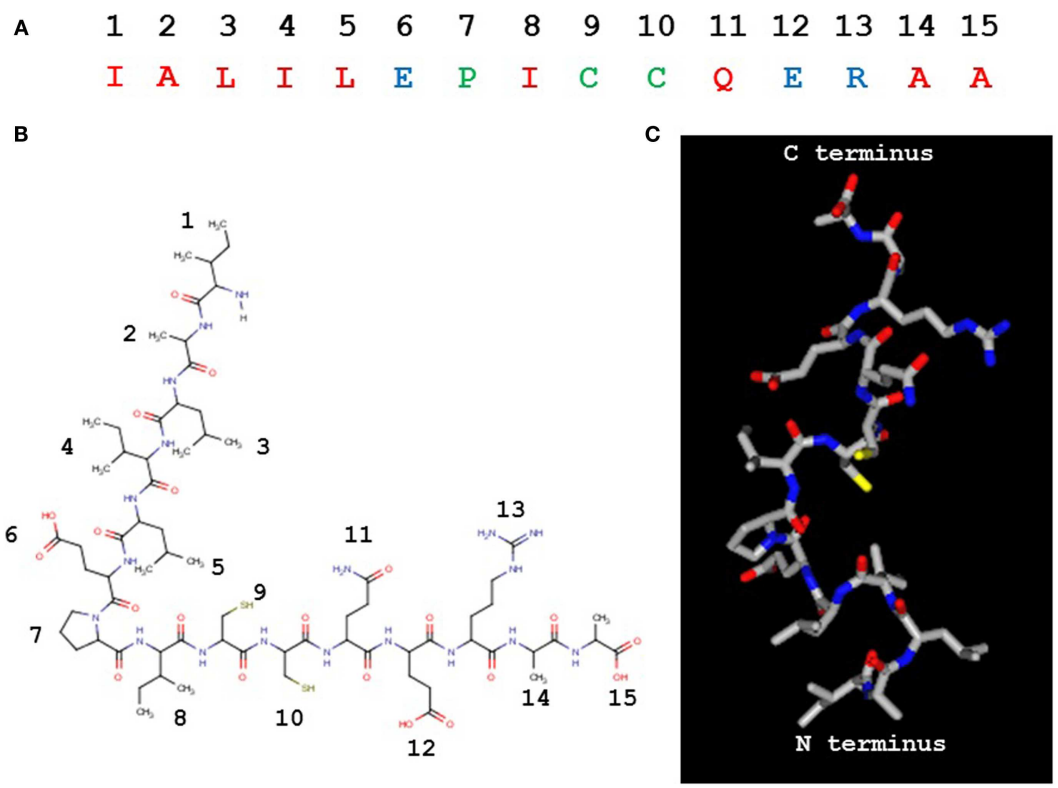

FIGURE 4 | Peptide inhibitors of complement C1 derivative PA. **(A)** The 15 residue PA molecule is made up of 9 hydrophobic (red), 3 polar/charged (blue), and 3 intermediary (green) amino acid residues. **(B)** A linear version of the PA molecule illustrating the amino acid side chains. **(C)** A structural model of PA. **(B,C)** Created in MarvinView.

ex vivo. Administration of the acetylated PA molecule resulted in *in vivo* complement suppression up to 24 h post-injection (27). These findings demonstrate that PIC1 molecules have excellent potential for pre-clinical testing in small animal models of antibody-initiated complement disease.

DEVELOPMENT OF A RAT MODEL OF AIHTR

A simple yet elegant model of complement-mediated AIHTR in rodents has been previously reported (30, 31). In this mouse model, developed in the laboratory of Dr. K. Yazdanbakhsh (32–34), human RBCs fluorescently labeled with the dye PKH-26 were transfused i.v. via tail vein and complement-mediated hemolysis of the transfused cells analyzed. In this mouse strain, natural antibodies directed against antigens on the human RBCs initiated complement activation leading to rapid lysis over 120 min. Soluble complement receptor 1 (sCR1) or derivatives of this molecule were demonstrated to temporarily inhibit hemolysis as well as C3 and C4 deposition on the transfused human RBCs (32). In the past few years, sCR1 (also known as TP-10) has been explored in clinical trials of human diseases; however, these trials have been discontinued for various reasons. In order to test PIC1 in a pre-clinical model of AIHTR, we have recently developed a Wistar rat transfusion model (35). The rationale for utilizing the rat is threefold: (i) the larger size of the rat provides adequate blood volume to perform multiple blood draws for analysis post-transfusion, (ii) extracted mouse serum is notoriously difficult to use in *ex vivo* hemolytic assays (36), and (iii) while human RBCs are identical in size to rat RBCs, they are twice the size of mouse RBCs and thus may have difficulty in transiting the narrow capillaries of the mouse (37). Wistar rats are also thought to have natural antibodies against human erythrocytes (38).

To establish this model, we first determined *in vitro* that Wistar rat serum lysed human RBCs in an antibody-initiated, classical complement pathway dependent manner by utilizing complement sufficient or complement-deficient Wistar rat serum in the presence and absence of naturally occurring anti-human RBC antibody (35). This was achieved by coating human AB RBCs with complement-deficient Wistar rat serum that contains antibodies to the human RBCs. When the antibody coated RBCs were exposed to antibody deficient, complement sufficient Wistar rat serum, the RBCs were lysed in a dose-dependent manner (i.e., increasing the amount of antibody on the surface of the RBCs increased lysis by rat serum). Thus, lysis required both anti-human erythrocyte antibodies from the Wistar rat serum and activatable rat complement. Using various buffers, we demonstrated that the lysis of the RBC *in vitro* was due to classical pathway activation. To study the role of complement in acute intravascular hemolysis *in vivo*, Wistar rats were treated either with or without cobra venom factor (CVF) to deplete complement activity. PHK-26 labeled or unlabeled human AB RBCs were then injected

into both groups of rats, followed by serial blood draws up to 2 h. Venous blood clearance and lysis of transfused RBCs at each time point was measured by flow cytometry (FITC labeled anti-human CD235a was used to detect unlabeled human cells) and spectrophotometry for free hemoglobin. Transfusion of human RBCs into rats showed significantly less hemolysis in the CVF group versus untreated group for both PHK-26 labeled and unlabeled cells by both flow cytometry and spectrophotometry (35). RBC sequestration was determined in the liver, spleen, and kidney by immunohistochemistry 2 h post-transfusion and demonstrated no quantitative difference for extravascular RBCs in the tissues between the two groups for the liver and spleen (35). However, intravascular human RBCs in the kidney glomeruli were different between the two groups, consistent with the flow cytometry and spectrophotometry data. Given these findings, we believe this simple rat model is ideal for testing novel inhibitors of classical pathway activation such as PIC1 for the prevention and treatment of AIHTR. Experiments to test PIC1 in this model are currently underway in our laboratory.

COMPLEMENT INHIBITORS FOR TRANSFUSION MEDICINE APPLICATIONS

Acute hemolytic transfusion reactions occur in almost one-fifth of total transfusions, with life-threatening reactions in approximately 0.5% (39). The clinical presentation of acute hemolytic transfusion reactions is broad, from the transfusion recipient who exhibits only mild and transitory signs and symptoms, to more serious cases, with shock, disseminated intravascular coagulation, renal failure, and death. The most severe reactions result from transfusion of incompatible RBCs from donor to recipient leading to rapid intravascular complement-mediated hemolysis. As there are no specific interventions to directly mitigate the effects of acute hemolytic transfusion reaction, only safeguard measures to reduce the risk of an incompatible transfusion are currently employed. Although current safeguards make ABO incompatible transfusions rare in the developed world, AIHTR remains a very high-risk threat for patient populations receiving frequent transfusions. Specifically, individuals with sickle cell disease and severe thalassemias requiring frequent transfusions become "difficult to cross match" due to the accumulation of antibodies against minor antigenic determinants on erythrocytes. In situations of life-threatening anemia (i.e., inadequate oxygen carrying capacity) these patients are not infrequently transfused with the "least incompatible" erythrocytes, because some degree of agglutination occurs with the cross match of all available units. These transfusions are extremely high risk for causing AIHTR, but must be done because the alternative is death by shock. An ideal therapeutic intervention for these individuals would be prophylactic treatment prior to transfusion with "least incompatible" erythrocytes, to decrease the risk of AIHTR. The ability to prevent AIHTR for these patients remains an important unmet medical need. As we have described in this review, the classical pathway of complement plays a central role in AIHTR. Thus, therapeutic inhibition of this system presents a viable strategy to mitigate and prevent AIHTR. Below, we discuss various complement inhibitors, both marketed and in development, and their potential for transfusion medicine and other blood disorders.

MARKETED COMPLEMENT INHIBITORS

C1-inhibitor

C1 esterase inhibitor (C1-INH) is a serine protease inhibitor that was initially demonstrated to regulate the C1 complex by binding and releasing the C1s–C1r–C1r–C1s tetramer from C1q after activation has occurred (40, 41). C1-INH has a broad specificity and also regulates the MBL–MASP2 complex of the lectin pathway of complement as well as serine proteases of the fibrinolytic, coagulation, and kinin systems. C1-INH has been successfully marketed as a replacement therapy for hereditary angioedema (HAE) in which the afflicted individual makes inadequate or defective C1-INH protein. While it is appreciated that HAE is primarily a kinin disease (42), C1-INH has been demonstrated to inhibit complement-mediated disease in several animal models and is currently in clinical trials for trauma and kidney transplantation [reviewed in Ref. (43)]. Recently, a study reported high dose C1-INH concentrate could prevent complement-mediated lysis of RBC in an elderly patient with autoimmune hemolytic anemia (43). While application of C1-INH has potential in complement-mediated disease, aside from HAE, super-physiological amounts of C1-INH are required to see a clinical effect when C1-INH is normally expressed in humans. Infusing high concentrations of C1-INH is of potential concern given C1-INH's promiscuous inhibition of other serine proteases (43); however, such high doses may be tolerable in an acute intervention setting. While recombinant C1-INH is now available, the purified version of human C1-INH is a blood product requiring extensive purification from large numbers of donors, resulting in an extremely expensive medication that still carries a residual risk of transmitting blood-borne pathogens. The results of the two clinical trials to determine the efficacy of C1-INH are eagerly awaited.

Nafamostat

Nafamostat is a synthetic serine protease inhibitor of C1s and other proteases currently marketed for pancreatitis. As is the case with C1-INH, nafamostat inhibits serine proteases other than C1s; thus, it is unclear if its clinical effects can be attributed to suppression of the complement system (44) as opposed to its known anticoagulant properties (45). Currently, nafamostat is only approved in a limited market (Japan and Korea). Other small molecule inhibitors of C1s have also been characterized (46); however, to our knowledge, the role of these inhibitors in transfusion-related indications has not been reported.

Eculizumab

Eculizumab is a highly specific, humanized monoclonal antibody that inhibits activation of C5 by the C5 convertase preventing C5a release and MAC formation. Eculizumab has been marketed for the orphan disease paroxysmal nocturnal hemoglobinuria (PNH) preventing the intravascular lysis of RBC that lacks the membrane bound complement regulators CD55 and CD59 (47). Eculizumab is also marketed for atypical hemolytic uremic syndrome (aHUS) (48) and is currently in clinical trials for other indications (44). Eculizumab has also been reported to reverse antibody-mediated complement activation in a case of ABO incompatible kidney and pancreas transplantation (49), and kidney-alone transplantation (50) as well as other off-label uses.

Eculizumab is very effective for individuals for PNH; however, it is currently one of the most expensive drugs on the market as a result of its high cost of production and its orphan status. Additionally, since it blocks complement activation at the level of C5, patients utilizing this drug are at increased risk of invasive meningococcal infections and therefore require vaccination. Another drawback for antibody-mediated complement disease indications is that eculizumab acts downstream of C3 and therefore cannot prevent classical pathway generated C3 activation products such as C3a formation or C3b/iC3b opsonization of host cells (e.g., CR1/CR3-mediated clearance of C3b/iC3b-coated RBC in the liver and spleen). This may limit eculizumab's potential for antibody-mediated, transfusion medicine related indications.

COMPLEMENT INHIBITORS CURRENTLY IN DEVELOPMENT

A number of complement inhibitors are currently in various stages of pre-clinical and clinical development [please see Ref. (44) for a thorough review]. As one would expect, the compounds currently under pharmaceutical development target a variety of molecules of the complement system including effector molecules and receptor proteins (C5aR, C3, C5, etc.). Given the ubiquitous role of complement in a variety of diseases, these inhibitors have a wide number of indications from transplantation to cancer. Most of these compounds are of the following classes: protein, antibody, or small molecule. Interestingly, there are currently only two peptide compounds in pharmaceutical development. One of the peptides that shows great promise is compstatin, a cyclical peptide that binds C3 preventing C3 activation of all three complement pathways (51). Analogs of compstatin are in various stages of pre-clinical and clinical development for indications such as age-related macular degeneration (AMD), PNH, aHUS, dense deposit disease (DDD), transplantation, and hemodialysis (44). Recently, compstatin analogs have been demonstrated to inhibit hemolysis of PNH RBCs in an *in vitro* system (52), indicating that compstatin may be useful in this RBC disorder. Additionally, compstatin has a significant theoretical advantage over eculizumab by virtue of its ability to block C3 cleavage and thus preventing C3b/iC3b opsonization of RBC leading to extravascular sequestration and hemolysis. While pharmacological blockade of C3 by compstatin positions this molecule as a potent inhibitor of complement, complete shutdown of this critical arm of innate immunity may lead to an increased risk of infection, whether this is a potential issue will become evident as clinical testing of compstatin progresses.

Recently, a mouse monoclonal antibody that targets C1s, known as TNT003, has been demonstrated to prevent complement deposition and RBC destruction via phagocytosis in an *in vitro* assay of CAD (53). TNT003 is currently under clinical development by True North Therapeutics, Inc., as a first-in-class compound designed to specifically inhibit the classical complement pathway. Dosing of TNT009, the humanized monoclonal analog of TNT003, in cynomolgus monkeys demonstrated that a single i.v. injection at 30 mg/kg inhibited the *in vivo* generation of C4a, as determined by ELISA. *Ex vivo* analysis of serum taken from animals receiving this single dose inhibited the hemolysis of IgM-coated sheep RBCs and the deposition of C3b on these cells, as assayed by FACS (54). TNT009 is expected to undergo phase 1 clinical trials in 2015 with an umbrella trial in Europe to test the efficacy of TNT009 in multiple complement disorders (54). The efficacy of TNT009 in clinical trials will be exciting to watch unfold.

PEPTIDE INHIBITORS OF COMPLEMENT C1

The fact that several complement inhibitors are currently in pre-clinical and clinical development is a testament to the central role that complement plays in a variety of inflammatory disorders. While the classical pathway of complement instigates antibody-mediated destruction of human RBCs in AIHTR, there is currently no therapeutic treatment for this condition. As outlined in this article, we propose that PIC1 might be an ideal molecule to prevent and treat AIHTR for the following reasons: (i) PIC1 inhibits at the level of the first complement component of classical pathway, C1. Inhibiting classical pathway activation at this step would prevent complement activation at initiation, thus, preventing amplification and downstream complement effector molecule generation (C3a, C5a, MAC, etc.) (**Figure 5**). (ii) PIC1 binds to the CLR of C1q and MBL. This is opposed to C1-INH and other small molecule serine protease inhibitors, which are known to non-specifically inhibit serine proteases outside of the complement system. Given that PIC1 possesses a differential mechanism of interaction with C1q that does not involve binding to catalytic sites of serine proteases, a higher specificity is expected. (iii) PIC1 does not inhibit the critical immune surveillance functions of the alternative and terminal complement pathway. While inhibitors such as compstatin can potently inhibit C3, effectively shutting down all three complement pathways, this has the potential to predispose individuals to an increased risk of infection. This has been documented for eculizumab that inhibits C5a formation and thus terminal pathway activation increasing the risk of infection by neisserial pathogens. (iv) PIC1 is unique in nature. This is opposed to C1-INH and soluble versions of known complement regulators that are produced endogenously in humans. Thus, molecules such as C1-INH and sCR1 may theoretically require very high doses to achieve a clinical effect. This remains to be determined, however, especially as concerns sCR1, which by virtue of its presence in

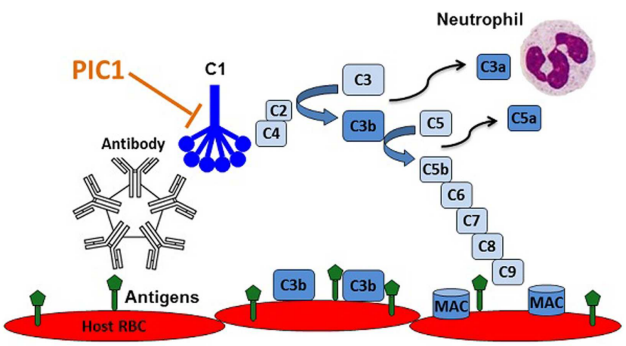

FIGURE 5 | Recognition of RBC antigens on recipient cells by host derived natural antibodies initiating classical complement via C1 leading to opsonization of host cells with C3b, anaphylatoxin C5a recruitment and activation of neutrophils, and cell lysis by membrane attack complex (MAC) pores. PIC1 blocks C1 activation at the first step of the amplification cascade.

solution may have additional effects compared to the endogenous membrane bound form. C1-INH and sCR1 appear efficacious in pre-clinical animal models; their efficacy in human trials is currently under evaluation. (v) PIC1 is cheap and easy to produce by virtue of it being a synthetic small peptide. This is in contrast to C1-INH and eculizumab, which are large molecules requiring extensive purification and high production costs. Both these molecules are currently in the top five most expensive drugs in the marketplace for their orphan disease indications, HAE (C1-INH), and eculizumab (PNH and aHUS) (55).

A potential concern for therapeutic inhibition of the classical complement pathway with molecules such as PIC1 or TNT003 is that long-term, systemic inhibition of C1 in humans could pose a risk for developing systemic lupus erythematosus (SLE). Humans lacking functional C1q have been demonstrated to acquire SLE due to the critical role of the classical pathway in maintaining homeostasis through the clearance of cellular debris and immune complexes (7). In the context of an acute disease target such as AIHTR, classical pathway inhibition would not be expected to be a major concern for development of SLE. However, systemic, long-term use of classical pathway inhibitors for chronic disease processes would need to be carefully scrutinized in this regard.

Further development of PIC1 is necessary to test this compound in animal models of classical pathway mediated disease such as AIHTR. To this end, a high potency, water soluble PIC1 derivative has been identified and dose ranging studies in rats are currently underway. Important parameters such as pharmacodynamics, pharmacokinetics, and toxicity of this derivative will be established in these studies. Next, proof-of-concept experiments in the rat model of AIHTR will be performed. Extended dosing and toxicity studies in rats and in a second species (e.g., cynomolgus monkeys) will then need to be completed. This will set the stage for submission of an investigational new drug (IND) application to the FDA under an orphan drug designation. This approach has been successfully utilized for the complement inhibitors eculizumab and C1-INH.

In the US, death from acute hemolytic transfusion reactions has decreased significantly due to numerous safety checks; however, accidental transfusion of incompatible blood between donor and recipient continues to occur due to failure of screening tests or human error (56). Ensuring safe and compatible blood products can be particularly challenging in certain instances such as during emergencies, in a mass casualty setting, or when the supply of blood is limited. Moreover, patients who require frequent blood transfusions may develop multiple antibodies against minor determinants on RBC surfaces decreasing the likelihood that compatible blood can be rapidly identified (56). This is particularly true for patients with sickle cell disease and other individuals suffering from severe chronic anemia requiring many transfusions over their lifetime leading to their development of a wide range of alloantibodies against numerous minor and major erythrocyte determinants (15, 16, 57). The development of these alloantibodies can progress to the point where compatible blood may not be identified in a timely manner, requiring the use of a "least incompatible" RBC unit. Different therapies have been adopted in attempts to minimize the risk from these "least incompatible" units; for example, a "mini-transfusion" challenge can be utilized to try to identify those units, which may lead to a life-threatening complement-mediated acute intravascular hemolytic event before the entire unit is transfused (18). Red cell antigen-sensitized patients, such as those with sickle cell disease or thalassemia, constitute a tremendous challenge for clinicians and the transfusion service particularly in the emergent setting (58). Given the acute hemolysis and hemoglobinemia already occurring for sickle cell disease patients suffering a vasoocclusive crisis, the high risk for worsening their clinical condition with additional AIHTR hemolysis is especially worrisome. In this type of situation, pre-treatment with PIC1 would minimize the risk of AIHTR for sickle cell disease patients meeting a major unmet medical need in an under-served population.

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The role of the Lys628 (192) residue of the complement protease, C1s, in interacting with peptide and protein substrates

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The C1s protease of the classical complement pathway propagates the initial activation of this pathway of the system by cleaving and thereby activating the C4 and C2 complement components. This facilitates the formation of the classical pathway C3 convertase (C4bC2a). C1s has a Lys residue located at position 628 (192 in chymotrypsin numbering) of the SP domain that has the potential to partially occlude the S2–S2′ positions of the active site. The 192 residue of serine proteases generally plays an important role in interactions with substrates. We therefore investigated the role of Lys628 (192) in interactions with C4 by altering the Lys residue to either a Gln (found in many other serine proteases) or an Ala residue. The mutant enzymes had altered specificity profiles for a combinatorial peptide substrate library, suggesting that this residue does influence the active site specificity of the protease. Generally, the K628Q mutant had greater activity than wild type enzyme against peptide substrates, while the K628A residue had lowered activity, although this was not always the case. Against peptide substrates containing physiological substrate sequences, the K628Q mutant once again had generally higher activity, but the activity of the wild type and mutant enzymes against a C4 P4–P4′ substrate were similar. Interestingly, alteration of the K628 residue in C1s had a marked effect on the cleavage of C4, reducing cleavage efficiency for both mutants about fivefold. This indicates that this residue plays a different role in cleaving protein versus peptide substrates and that the Lys residue found in the wild type enzyme plays an important role in interacting with the C4 substrate. Understanding the basis of the interaction between C1s and its physiological substrates is likely to lead to insights that can be used to design efficient inhibitors of the enzyme for use in treating diseases caused by inflammation as result of over-activity of the classical complement pathway.

Keywords: complement, C1s, C4, substrate, protease, serine protease

INTRODUCTION

The complement system is of major importance in innate and adaptive immunity (1), but has also been shown to play a major role in several inflammatory diseases (2, 3). Understanding the precise mechanisms whereby the pathways of the system are activated is therefore likely to provide understanding of potential mechanisms to control the system. Once activated following the recognition and initial activation events of the classical pathway, C1s plays a major role in amplifying the initial recognition by cleaving and thus activating the C4 and C2 complement proteins that go on to form the C4bC2a C3 convertase complex (4, 5). It has recently been shown that the activation of the C1s protease is concomitant with the formation of an exosite on the serine protease domain (6, 7) that works in concert with a likely exosite on the CCP domains and the active site to efficiently catalyze the cleavage of C4 to yield the C4b and C4a fragments. In this regard, the interaction between C1s and C4 is likely to have similarity to the MASP-2-C4 interaction, which has been elucidated structurally (8) and biochemically (9–11). Given that the exosite interactions for the classical and lectin pathway proteases are somewhat similar, the focus falls on the active sites of the proteases in order to provide selective inhibitors of the pathways for use in controlling diseases in which they are involved.

The X-ray crystal structure of the activated CCP2-SP form of C1s (12) shows that the active site of the enzyme is quite "closed" and restricted from its surrounding environment, which most likely contributes to its high level of substrate specificity (13). During the cleavage of C4, C1s must accommodate a bulky P2 Gln into a relatively small S2 subsite. To date, there is no X-ray crystal structure of C1s with a peptide substrate bound in the active site, and therefore, it is unknown how the protease finds the capacity to accommodate such a large amino acid. The Lys628 (192 in chymotrypsin numbering) is a dominant feature of the active site, as it overlaps the entrance to the crucial S2–S2′ subsites of the enzyme (12) (**Figure 1**).

The K628 (K192 in chymotrypsin numbering) amino acid has been shown to be vital in many serine proteases. Only 14% of trypsin-like proteases in the human genome contain a lysine at position 192, while 60% contain glutamine and 5.4% glutamate (14). Previous studies in which serine proteases were mutated at

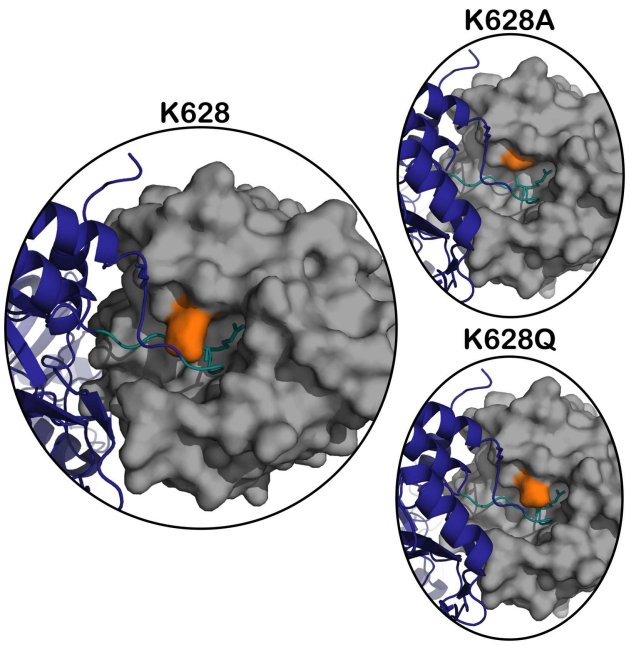

FIGURE 1 | Superposition of active C1s (gray) (PDB ID: 1ELV) in surface representation, on the structure of the MASP-2 – C4 complex (C4 in navy blue) (PDB ID: 4FXG) in cartoon representation. The position of K628 (orange) is highlighted with respect to the P4–P4′ cleavage loop of C4 (in teal, P1 arginine sidechain shown in stick). The panels on the right represent the structure in the same orientation with the K628 position mutated to an alanine (top panel) and glutamine (bottom panel).

position 192 have shown that mutation of this residue can have a variety of effects on the interaction of chymotrypsin-family serine proteases with substrates and inhibitors (14–16).

Here, we have mutated this residue in C1s to Gln and Ala in order to understand its role in the interaction with peptide substrates and C4. The results indicate that the Lys residue at this position in the protease plays a role in facilitating efficient cleavage of C4 in particular.

MATERIALS AND METHODS

PRODUCTION OF RECOMBINANT C1s AND MUTANTS

Recombinant human C1s and the K628Q and K628A mutants were expressed in *Escherichia coli* as insoluble proteins in the inclusion bodies as described previously (7). The enzymes were denatured and then refolded, following which they were purified by a combination of anion exchange and gel filtration chromatography all as previously described (7). All proteins were obtained in good yield and purity. The zymogen forms of the proteases were activated using immobilized C1r as previously described (7). The activated proteases were titrated using C1-inhibitor (CompTech, TX, USA) to yield the final active concentrations of each enzyme form.

CLEAVAGE OF THE REPLi COMBINATORIAL SUBSTRATE LIBRARY BY C1s ENZYMES

Wild type or mutant C1s forms (400 nM) were tested for their ability to cleave a combinatorial peptide substrate library (REPLi, Mimotopes, Clayton, VIC, Australia) containing 3375 different peptides arranged in 512 pools (17). The assays were conducted in fluorescence assay buffer (FAB) [50 mM Tris–HCl, 150 mM NaCl, 0.2% (w/v) PEG 8000, pH 7.4] at 37°C. Cleavage of the substrates was monitored by measuring the increase in fluorescence intensity from the MeOC fluorophores using 55 s cycles for 30 cycles, with an excitation wavelength of 320 nm and an emission wavelength of 420 nm. The initial velocity of the cleavage was indicated by the slope per unit time of the linear region of the curves.

CLEAVAGE OF PEPTIDE SUBSTRATES CONTAINING PHYSIOLOGICAL SEQUENCES

Assays were carried out in FAB at 37°C using final substrate concentrations in the range of 0.5–500 μM. The fluorescence quenched C4 P4–P4′ substrate (FQS) [2-aminobenzoate-GLQRALEI-Lys(Dinitrophenol)-NH_2] and coumarin substrates {C2 P3-P1 substrate [Z-LGR-aminomethylcoumarin (AMC)] and C4 P5-P1 substrate [Z-AGLQR-AMC]} were solubilized in 10% (v/v) dimethylformamide. The rate of increase of fluorescence in the presence of 400 nM C1s (wild type or mutant) was measured on a BMG Technologies FluoStar Galaxy fluorescent plate reader (BMG Labtech, Offenburg, Germany) using an excitation wavelength of 320 nm and an emission wavelength of 420 nm for the FQS and 360/460 nm for coumarin substrates. The initial reaction rate was estimated at a single concentration of enzyme from duplicate measurements over a range of substrate concentrations. In order to determine steady-state reaction constants [V_{max} (maximal velocity) and $K_{0.5}$ (half saturation constant)], the experimental results were fitted, using the GraphPad Prism Version 5.0 computer program (GraphPad Software, San Diego, CA, USA), to an equation describing positive cooperativity ($V = V_{max}[S]h/[S]h + [K_{0.5}]h$), which defines the relationship between reaction rate (V) and substrate concentration ([S]) when more than one binding site applies (18). The catalytic efficiency (k_{cat}) values were calculated as described previously (18).

CLEAVAGE OF C4

C4 (CompTech, TX, USA) at 1 μM (final concentration) was incubated with increasing concentrations of C1s or mutants (0–5 nM) for 1 h in FAB at 37°C. Reactions were stopped by the addition of reducing SDS-loading buffer and samples were incubated at 90°C for 5 min, then loaded onto 12.5% SDS-PAGE and electrophoresed. Gels were stained with Coomassie blue R-250 stain and destained. The cleavage of the C4 alpha band was analyzed using the gamma band as a loading control. A Typhoon Trio (488, 532, and 632 nm lasers) was utilized for densitometry analysis using the IQTL ImageQuant™ software (1D Gel Analysis) (GE Healthcare, Australia). The densitometry data for the disappearance of the alpha band of C4 was plotted against the log of the concentration of C1s used. The EC_{50} values were derived by fitting the data using non-linear regression to the following equation: $Y = Y_{min} + (Y_{max} - Y_{min})/\{1 + 10^{[(LogEC50-X)*h)]}\}$, where Y_{min} is the minimum Y-value, Y_{max} is the maximum Y-value, and h is the Hill slope. The analysis was carried out using GraphPad Prism, which output 95% confidence intervals for each value that indicated that the value obtained for the wild type enzyme was significantly different from those obtained for the mutant forms at

a 95% confidence level. The experiment was repeated three times with similar results obtained.

The time course of cleavage of C4 (1 μM) by C1s forms at 1 nM was derived by incubating the components for 0, 1, 2, 5, 15, 30, 60, and 120 min, following which the reaction mixtures were treated as described above. The disappearance of the alpha band was quantified by densitometry as specified above.

RESULTS

The REPLi peptide substrate library provides a rapid means of investigating the substrate specificity of a protease. We have previously characterized the substrate specificity of C1s using a phage display-based analysis (13), which yielded a highly cleaved sequence, YLGR-SYKV, consistent with the preference of this enzyme for a small amino acid, such as Gly, at P2 and a hydrophobic amino acid, such as Leu, at P3. The top ranked substrate for C1s in the REPLi library had the form K/R-S/T-I/L (**Figure 2**). Since C1s is highly specific for Arg residues at P1 in particular, we can assume this substrate would be cleaved after the K/R position. Since Gly residues occupy all positions other than those being varied in these substrates, this would be the residue found at P2 in this substrate. The strong preference of C1s for P2′ Leu and P1′ Ser residues has also been noted before and the sequence overall is similar to that found at the P1–P2′ of C1-inhibitor, with C4 also having a Leu at P2′.

Overall, the K628Q enzyme mutant was much more active against substrate pools in general than the wild type enzyme, while in general the K628A mutant was less active than the wild type enzyme. This was not true for substrates with a Pro residue at the likely P2 position, which the K628A mutant cleaved better than wild type in general, although less well than the K628Q mutant. The K628A mutant was also more active against the K/R-A/V-K/R pool than wild type. While it cannot be certain where the enzyme would cleave in this substrate, as there are two potential Arg residues that may constitute the P1 residue, there is a likelihood that the second K/R residue would lie within P2′, and thus, it appears that the absence of K628 favors cleavage of this substrate pool. In support of this, the wild type enzyme cleaved the K/R-A/V-D/E pool more rapidly than either mutant. In that case, the D/E residue must sit at P2′ and it appears that the positively charged K628 favors interaction with the oppositely charged D/E residue. Thus, it appears that the K628 residue might indeed be able to interact with the P2′ residues of substrates.

It is worthy of note that the enzyme essentially did not cleave substrates of the form K/R-K/R-I/L (the P1–P2′ sequence of C2), N/Q-K/R-A/V (the P2–P1′ sequence of C4), or I/L-N/Q-K/R (P3–P1 of C4). This indicates that the Lys residue found at the P1′ position in C2 and the Gln residue found at the P2 position of C4 are likely to be prohibitive for cleavage of substrates by C1s. However, the K628Q and K628A mutants of C1s also did not cleave these substrate pools, indicating that K628 does not play a crucial role in preventing interaction with such substrates.

The results obtained with the REPLi peptide substrate library suggested that the K628 residue of C1s plays a role in restricting the substrate specificity of C1s for peptide substrates. In general, the replacement of the Lys residue with the Gln residue found at this position in many other serine proteases increased the cleavage rate of the enzyme for many pools of substrates, while replacement with an Ala had the opposite effect. This general trend was also observed with the Z-LGR-AMC and Z-AGLQR-AMC peptide substrates, representing the P3–P1 and P5–P1 sequences of C2 and C4, respectively (**Table 1**). The $k_{cat}/K_{0.5}$ value for the K628Q mutant with Z-LGR-AMC was fivefold higher than that for wild type, while the same parameter for K628A was twofold decreased compared

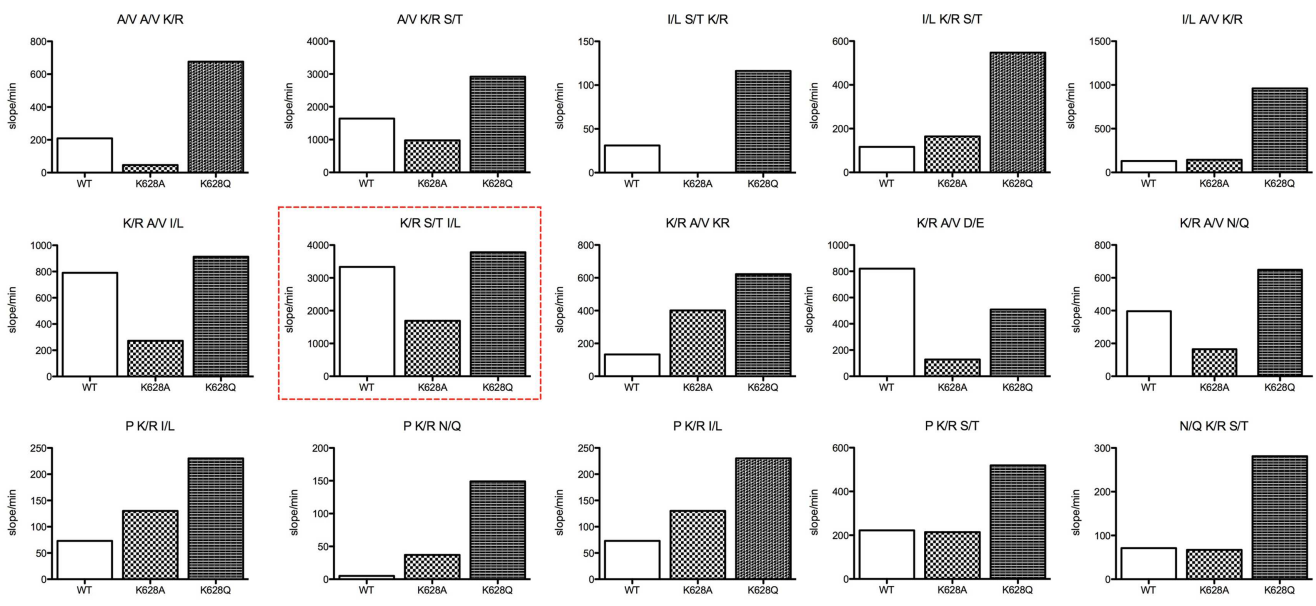

FIGURE 2 | Bar graphs of the initial velocity values for substrate pools of the REPLi combinatorial peptide substrate library incubated with wild type and K628 mutants of C1s. The identity of the three amino acid positions varied for each pool displayed is shown above each graph. Initial velocity values for wild type (empty bar), K628A (lighter hatched bar), and K628Q (dark hatched bar) are shown.

Table 1 | Kinetic parameters for cleavage of peptide substrates by wild type C1s and K628 mutants.

Substrate and parameter		Wild type C1s	C1s K628Q	C1s K628A
Z-LGR-AMC	$K_{0.5}$ (μM)	169 ± 7.9	76.2 ± 1.15	212 ± 13.8
	k_{cat} (s^{-1})	2.72	6.0	1.77
	$k_{cat}/K_{0.5}$ ($M^{-1}s^{-1}$)	1.6×10^4	7.9×10^4	8.3×10^3
Z-AGLQR-AMC	$K_{0.5}$ (μM)	245 ± 13.3	65.4 ± 1.91	109 ± 18.9
	k_{cat} (s^{-1})	35.33	116.5	1.3
	$k_{cat}/K_{0.5}$ ($M^{-1}s^{-1}$)	1.4×10^5	1.8×10^6	1.2×10^4
Abz-GLQRALEI-Lys(Dnp)	$K_{0.5}$ (μM)	13.8 ± 0.3	12.3 ± 0.49	12.0 ± 0.6
	k_{cat} (s^{-1})	1.14	1.17	1.3
	$k_{cat}/K_{0.5}$ ($M^{-1}s^{-1}$)	8.2×10^4	9.5×10^4	1.1×10^5
C4	EC_{50} (nM)	0.16	0.76	0.77

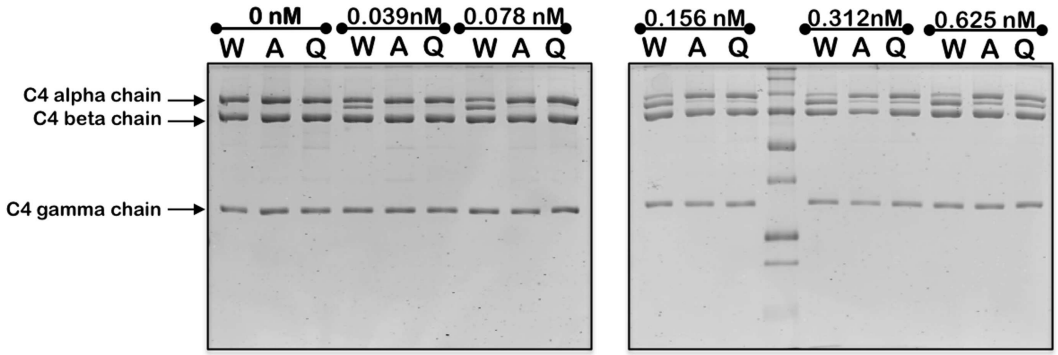

FIGURE 3 | Cleavage of C4 by increasing concentrations of wild type and mutant forms of C1s. The cleavage of C4 (1 μM) by the indicated concentrations of wild type (W), K628A (A), and K628Q (Q) forms of C1s was allowed to proceed for 1 h, following which the reaction was stopped by the addition of the loading buffer for SDS-PAGE. The alpha, beta, and gamma chains of C4 are indicated to the left. The cleaved remnant of the alpha chain appears as a band immediately below that of the alpha chain. The experiment was repeated three times with similar results.

to the wild type enzyme. The major determinant of the effects was the change to the k_{cat} parameter in each case. The $k_{cat}/K_{0.5}$ for the Z-AGLQR-AMC substrate was increased over 10-fold for the K628Q mutant, while that for the K628A mutant was decreased over 10-fold. Again, the changes to the k_{cat} parameter were the major effect noted.

Interestingly, different results were obtained with the Abz-GLQRALEI-Lys(Dnp) fluorescence quenched peptide representing the P4–P4′ sequence of C4. In this case, the $k_{cat}/K_{0.5}$ values for wild type versus the K628Q mutant were essentially the same, while the same parameter for K628A was slightly increased. This indicates that the addition of the prime side residues and the removal of a very bulky coumarin group from the P1′ position of the substrate had major effects on the interaction of the enzymes with the substrate, such that all were essentially equal in their ability to cleave the amino acid sequence found at the cleavage point within the C4 substrate.

Mutation of the K628 residue to either a Gln or Ala residue reduced the efficiency of cleavage by approximately fivefold (**Table 1**). Thus, at concentrations up to 0.3 nM, the wild type enzyme cleaved C4 much more efficiently than either mutant enzyme (**Figure 3**). The K628Q mutant also cleaved C4 considerably more slowly than the wild type enzyme (**Figure 4**). This was also found for the K628A mutant (results not shown).

DISCUSSION

In general, the results obtained with the REPLi library reinforced previous specificity data for this enzyme (13), although it was interesting to note that the I/L-S/T-K/R and I/L-A/V-K/R pools were not well cleaved by the wild type enzyme, somewhat contrary to previous data indicating a preference for Leu residues at the P3 position of substrates for C1s. The mutation of K628 to an Ala residue did not improve the activity of the enzyme against such substrates, but the K628Q mutant cleaved these substrate pools much better, indicating that the mutation to a Gln residue favored such substrates.

The results with the proposed P2′ residues of substrates are similar to what was noted for mast cell chymase, where a K192 residue mediated favorable contacts with negatively charged P2′ residues (19) and thrombin, where E192 restricted the specificity of the enzyme (20). Overall, however, it appears that K628 influences the substrate specificity of C1s at a number of different subsites, indicating that the amino acid sidechain might be interacting with amino acids at a number of different positions within a

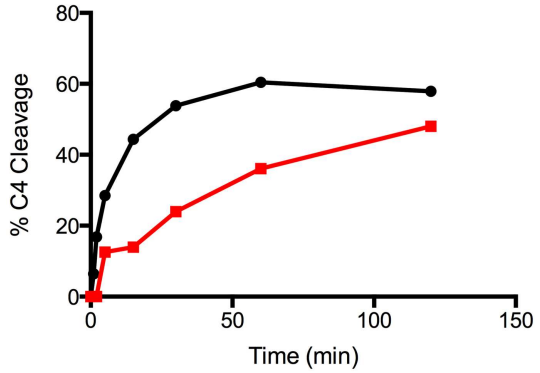

FIGURE 4 | Time course of the cleavage of C4 by wild type and K628Q forms of C1s. The cleavage of C4 (1 μM) by 1 nM wild type (black circles) and K628Q (red squares) forms of C1s was allowed to proceed for 0, 1, 2, 5, 15, 30, 60, and 120 min, following which the reaction was stopped by the addition of the loading buffer for SDS-PAGE. Following SDS-PAGE analysis, the disappearance of the alpha chain of C4 was quantified by densitometry and converted into a value indicating the percentage of cleavage.

peptide substrate. This indicates that the residue might be flexible in conformation, as was indicated in the structure of activated C1s previously solved (12).

We have previously shown (7) that the interaction of C1s with C4 can be assessed using analyses that derive the efficiency constant, EC_{50}, for cleavage of the substrate by the enzyme. Here, we obtained a somewhat lower EC_{50} for cleavage of the substrate by the wild type enzyme than that reported before [0.16 nM here versus 1 nM reported in Ref. (7)], but nevertheless these efficiency values are within range of each other. The enzymes also were found to cleave C4 more slowly than the wild type enzyme. Our data indicates that although the enzymes could cleave the P4–P4′ sequence of the substrate very similarly, the K628 residue clearly makes an important interaction with the C4 protein substrate that cannot be replicated by the polar Gln residue, suggesting that it is the positive charge of the K628 that is important in this regard. Since cleavage of a substrate that would be expected to bind within the active site was not changed (see Results for C4 P4–P4′ FQS), this would suggest that an interaction between K628 and a substrate residue external to the active site is occurring.

The amino acid found at the 192 position of chymotrypsin-family serine proteases has been shown to be critical in the regulation of interactions with substrates and inhibitors (16, 19, 20). The K628(192) residue of C1s partially occludes the active site by restricting access to the S1 pocket and therefore changes in the interaction with substrates were anticipated. However, it should be noted that in the structure obtained for the active C1s CCP2-SP, K628 is partially disordered, which may suggest the amino acid is quite dynamic in nature (12). It is therefore possible that in the case of the interaction between C1s and C4, the K628 residue is playing a role outside of the active site and interacting with a negatively charged residue of the substrate.

We have previously shown that a positively charged exosite located on the serine protease domain of C1s interacts with a negatively charged site on C4 to yield efficient binding and cleavage of the substrate molecule by the enzyme (7). It is unlikely that it is the same negatively charged site of C4 that is interacting with the Lys628 residue of C1s, but it does suggest that the exosite interactions of the enzyme extend somewhat beyond the originally defined four amino acids of C1s. In the structure of the related MASP-2 enzyme in complex with C4 (8), the Arg residue at the 192 position of this enzyme (630 in numbering of residues in the MASP-2 structure) lies very close to the loop that is cleaved in C4, but it appears to be making little direct contact with the substrate, thus examination of this complex does not provide any obvious clues as to the possible interactions that the K628 residue of C1s might be making. It should be noted that the active site architecture of C1s is markedly different at this position compared to MASP-2, however (12, 21). Modeling of the C1s into the same position as the MASP-2 in this structure (**Figure 1**) does not provide any further insights, suggesting that the residue may have to change orientation considerably to be able to achieve the new interactions suggested by the data obtained here. In the structure of the zymogen form of C1s (6), this residue faces inwards and away from possible substrate contacts, suggesting that it too is structurally removed from possible substrate interactions, in common with the "main" exosite of the serine protease domain of C1s, which is structurally altered in the zymogen form so that interactions with substrates are minimized in this form of the enzyme.

The studies carried out here give additional insight into the interactions mediated by the K628 residue of C1s. They suggest that in the specific case of the C4 substrate, the residue is playing a role outside of the active site interactions with substrates. However, equally it is clear that the residue can affect the functioning of the enzymes active site in cleaving peptide substrates and thus it must be considered when designing molecules to interact with the active site of the enzyme in order to inhibit it. Such molecules would be expected to have uses in counteracting a number of inflammatory diseases.

AUTHOR CONTRIBUTIONS

Lakshmi Carmel Wijeyewickrema helped to design the study, planned and carried out analyses, prepared the figures, and helped to write and revise the manuscript. Renee Charlene Duncan helped to design the study, planned and carried out analyses, prepared the data in publishable format, and helped to write the manuscript. Robert Neil Pike played a major role in designing the study, wrote much of the manuscript, and revised it for final submission.

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Deciphering the fine details of C1 assembly and activation mechanisms: "mission impossible"?

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The classical complement pathway is initiated by the large (~800 kDa) and flexible multimeric C1 complex. Its catalytic function is triggered by the proteases hetero-tetramer C1r2s2, which is associated to the C1q sensing unit, a complex assembly of 18 chains built as a hexamer of heterotrimers. Initial pioneering studies gained insights into the main architectural principles of the C1 complex. A dissection strategy then provided the high-resolution structures of its main functional and/or structural building blocks, as well as structural details on some key protein–protein interactions. These past and current discoveries will be briefly summed up in order to address the question of what is still ill-defined. On a functional point of view, the main molecular determinants of C1 activation and its tight control will be delineated. The current perspective remains to decipher how C1 really works and is controlled *in vivo*, both in normal and pathological settings.

Keywords: classical complement pathway, C1 complex, C1r, C1s, C1q, serine protease activation, complement-dependent cytotoxicity, X-ray structures

INTRODUCTION

In 1897, at the very early period of nascent immunology, the Nobel price winner Jules Bordet discovered a heat-sensitive serum effector triggered by immune complexes and absolutely required for the lysis of Ab-coated erythrocytes or bacteria. At that time, it was named "alexine." As discovered later on, this effector mechanism is very complex, involving many proteins, namely the complement system (C) triggered via the classical pathway (CP) (1, 2) (see **Figures 1A,B**). Deciphering the fine structural mechanisms governing this CP-activating function of the first C component C1 remains experimentally difficult and has progressed through iterative steps, which will be briefly summarized here.

Why is it important to decipher C1 structure and C activation mechanism? One obvious aim is to improve the C1-mediated effector mechanism in antibody therapeutics (8). C1 plays indeed a crucial role in the efficient elimination of Ab-coated targets, as confirmed by the disease susceptibility of patients affected by the deficiency in components C1q, C1r, C1s, and C4, all involved in the CP activation (9). Another hallmark of these deficiencies is the very large propensity to develop autoimmune diseases such as lupus erythematosus, which underlines that other essential functions are provided by the CP activation (9–13). On the other side, non-physiological activation of the CP or interferences by foreign substances such as carbon nanomaterial (14, 15) or a defective control of CP activation can also be strongly detrimental. Such undesirable activations can happen for example in cases of transplantation, neurological disorders, and rheumatoid arthritis (16) and thus new strategies to specifically inhibit the CP are awaited. On a more general standpoint, the functional impact of the complement system appears now far broader and more essential than initially assumed (17, 18).

INITIAL STUDIES AND FIRST LOW RESOLUTION FUNCTIONAL C1 MODELS

Very active pioneering investigations were performed during the 1963–1987 period (1–3, 19). The sequences of the C1q, C1r, and C1s subcomponents, their fixed (C1q:C1r2s2) stoichiometry, as well as the calcium-dependency of the interaction between C1r and C1s have been deciphered. Biochemical experiments revealed that C1r and C1s are sequentially activated (**Figure 1A**) and their unique Arg-Ile activation cleavage site has been precisely identified (3). In both cases, a disulfide bridge maintains a covalent link between the catalytic serine protease (SP) domain and the preceding modules. Careful protein biochemical analyses detailed the numerous C1q post-translational modifications such as proline and lysine hydroxylations and hydroxylysine glycosylations, which were mainly confirmed recently (20). The main functional domains were isolated by limited proteolysis of the serum-derived proteins and their shape studied by several biophysical methods such as small angle X-ray or neutron scattering and electron microscopy (21–23) (see **Figure 1C**). C1q is a very flexible 450 kDa molecule, partly stabilized by the associated protease tetramer (24). Catalytic and interaction domains were identified for each C1r and C1s protease (**Figure 1C**). In an apparent paradox, a very elongated shape was observed by neutron scattering for the protease tetramer in solution (larger maximum radius of gyration Rg of 17 nm) in contrast to the measures for C1q (Rg of 12.8 nm) and for the C1 complex (Rg of 12.6 nm), which suggested a substantial conformational change of the tetramer and/or C1q upon association (**Figure 1C**) (3, 25). The other intriguing feature was about the symmetry level inside the complex since the C1q hexamer associates with a proteases tetramer (19, 24). Several "low resolution" models were proposed for C1 at that time, the main

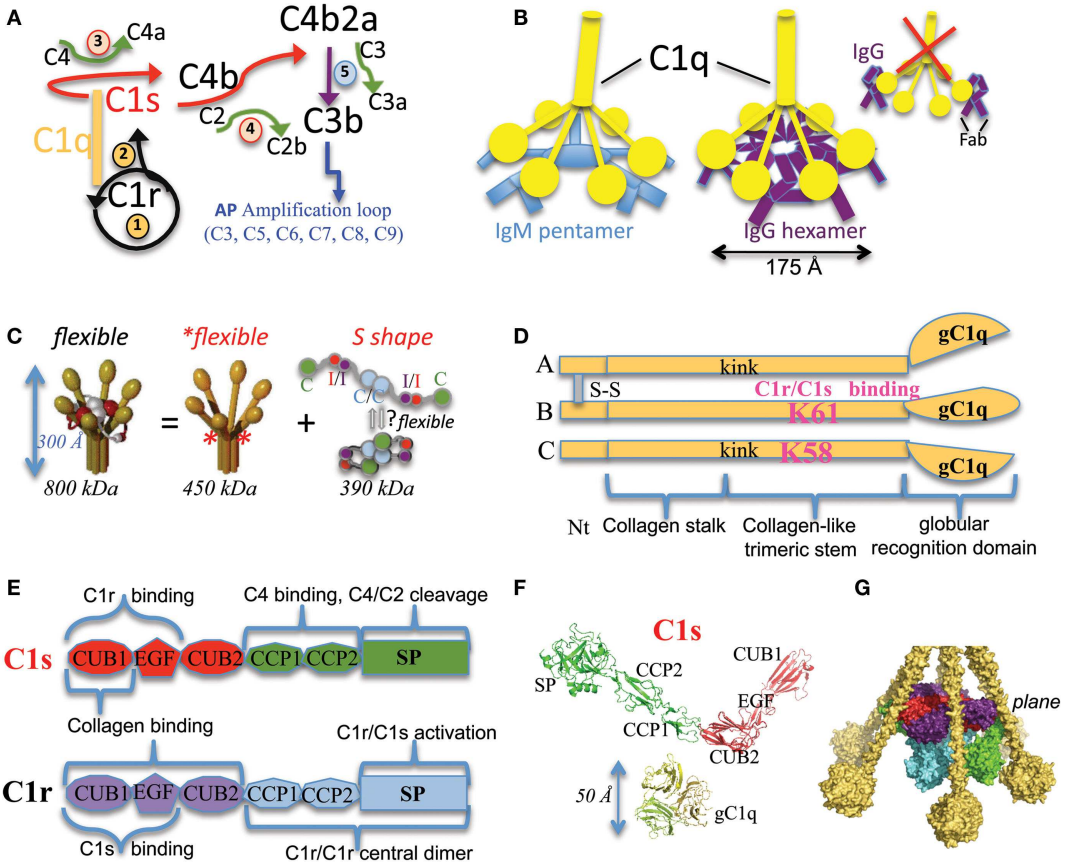

FIGURE 1 | Functional and structural elements of the CP activation. **(A)** The main steps of the complement activation cascade through the CP. The multimeric C1q molecule is associated to the C1s–C1r–C1r–C1s tetramer. When C1q binds to an activating target surface, a conformational change triggers the auto-activation of the associated C1r protease (converting the pro-enzyme into an activated form, black circular arrow), which then activates C1s (black arrow). C1s activates C4 and C4b-bound C2 (red arrows), leading to the assembly of the classical C3 convertase C4b2a. Green arrows are used for the activation cleavage of C2, C3, and C4, with the release of a small fragment. Details of the consecutive AP amplification loop are not given for sake of clarity. It involves C3–C9 components and mediates rapid opsonization, signaling events, as well as eventually formation of the lytic pore. The initial steps are numbered from 1 to 5. The first two steps occur inside C1 and depend on C1q conformational change and the consequent C1r activation. Steps 3 and 4 depend on C1s proper positioning and catalytic activity. **(B)** Current hypothetical schemes on similar interaction modes between C1q and IgM or IgG hexamers, the best CP activators. The new scheme proposed for IgG is in contrast with the traditional old scheme (right) depicting one C1q molecule interacting with two distant IgG molecules, each antigen-bound through its two Fab arms. **(C)** The "C1 paradox" and initial low resolution C1 models. C1 is a 30 nm high multimer resulting from the association of the flexible recognition protein C1q with the flexible C1s–C1r–C1r–C1s tetramer, which appears more elongated (S extended shape) in solution than in the complex (thus the initial "paradox"). C1q (yellow) has a hexameric shape, built from 18 chains. Interaction (I) and catalytic (C) domains of C1r and C1s are labeled and colored on the right side. The asterisks show the position of flexible hinges in C1q. The low resolution model on the left and the proposed tetramer conformational equilibrium on the right are derived from (3). **(D)** Modular structure of each C1q chain type. A, B and C chains associate as a hexamer of ABC heterotrimers. Kink indicates the position of disruptions in the triplets occurring only within collagen-like sequences of the A and C chains and probably inducing flexible hinges. The disulfide bridging between chains A and B is illustrated. The C chain has no covalent link with A and B chains, but covalently associates pairs of ABC trimers through a C–C disulfide bridge. The two lysines crucial for C1 assembly are shown in pink. **(E)** Modular structure and associated functions of C1r and C1s. The catalytic domain includes the C-terminal serine protease (SP) domain as well as the preceding Complement Control Protein (CCP or sushi) modules. The interaction domains of C1s and C1r involve their N-terminal CUB–EGF–CUB modules. The corresponding functional implications are mentioned. The same color coding is used in **(F,G)** and in the right panel of **(C)**. "CUB" means initially found in Complement C1r and C1s, Uegf and BMP-1. **(F)** C1 is a large complex made of small building blocks of (mainly) known structures. The displayed C1s is a composite structure obtained after superposing the PDB structures 1ELV (4) and 4LMFA (5) onto 4LOT (5) (see details in Table S1 in Supplementary Material). The color code used is the same as in **(C,E)**. The chains ABC from the C1q globular domain [2WNV (6)] are shown on the same scale. **(G)** Example side view of a partial composite C1 model, refined using the results of differential accessibility in C1q and C1 using chemical lysine labeling followed by mass spectrometry (7). The C1r and C1s proteases interact with C1q through their interaction domains aligned on the same plane (which corresponds to the position of LysB61 and LysC58 in C1q). This part of the model is mainly confirmed by recent complementary experimental studies (8). The position of the catalytic domain of C1s is more uncertain and probably variable.

differences being the speculations about its activation mechanism and on how the proteases are tightly packed inside C1, and whether they are fully kept inside the C1q cone or not (3, 19, 24, 26).

THE MAIN MOLECULAR PLAYERS INVOLVED IN C1 ACTIVATION AND ITS TIGHT CONTROL

The C1r and C1s proteases are produced as inactive precursors (called zymogens), and thus need to be activated "on the spot" by a specific Arg-Ile proteolytic cleavage in response to a triggering signal. This activating cleavage induces a conformational rearrangement, as classically described for the proteases of the trypsin-like family. C1-inhibitor, a protease inhibitor of the serpin family, exerts the main physiological control on these C1r and C1s proteases activity, by both inhibiting their activation and dissociating them from activated C1. C1 auto-activation can be observed *in vitro* in the absence of C1 inhibitor or through heating, which induces large conformational changes and also probably kills the C1-inhibitory effect (19). The adverse effects related to uncontrolled C1 activation are thus mainly linked to unbalanced C1-inhibitor control. C1-inhibitor is a multipotent serpin, controlling also some proteases of the fibrinolytic system, and contact/kinin system of coagulation in addition to the C1r, C1s, and MASP complement proteases and thus its deficiency leads to severe diseases such as hereditary angioedema (27).

IgM or IgG immune complexes are the best physiological C1 activators identified to date, especially in the presence of C1-inhibitor. Although it has been known for long that C1q binds to IgG Fc domain, and that activation requires multivalent binding, the details of how this can happen had remained poorly understood (8). IgG mutations are known to strongly influence C1q binding and C activation (28–31). Of note, these mutation studies did not fully confirm the originally predicted E-x-K-x-K IgG C1q-binding consensus motif (28), which remains, however, still used by some teams as a C1q-binding predictive tool.

A recent study has shown how IgG surface clustering through Fc-dependent hexamers could lead to very efficient C1 activation (8) (**Figure 1B**). Interestingly, this mode of hexameric clustering is far more similar to the pentameric/hexameric IgM assembly than to what was traditionally proposed (**Figure 1B**). It has long been described in text books that C1 activation involves binding to at least two IgG molecules, each one bound to the surface through its two Fab segments (**Figure 1B**). In contrast, in the recently proposed hexameric IgG assembly, each IgG seems to have only one Fab arm on the target surface, the other Fab arm lying on the same central plane as the clustered Fc platform (8). This recent breakthrough brings new clues about how to enhance the complement-dependent cytotoxicity of IgG, since the E345R mutation was described as a general C1 activation enhancer for all IgG isotype variants (8). The recent structure of the deglycosylated IgG4 Fc further supports this hypothesis of a possible generic hexameric Fc assembly, which is stabilized by this E345R mutation (32). The IgG1 and IgG4 Fc form quite similar hexameric rings of 175 Å diameter, which is of the same range of magnitude as the 180 Å diameter estimated for the comparable IgM Cμ3–Cμ4 hexameric platform (32). Local differences are observed between the different IgG isotypes in their hexameric interface composition and surface loop conformations (32). Of note, the IgG4 homologous C1q-binding loop is flexible, with at least two different conformations observed. The major conformation observed in native IgG4 prevents C1q binding, which correlates with the strongly reduced level of CP activation by native IgG4 hexamers (32).

CURRENT STRUCTURAL KNOWLEDGE ON C1 BUILDING BLOCKS AND KEY PROTEIN–PROTEIN INTERACTIONS

Although the first C1r crystals were obtained in 1981 [cited in Ref. (26)], X-ray crystallography analyses were initially limited, probably because of molecular flexibility. The C1 complex and most of its components look indeed very flexible (**Figure 1C**). A dissection strategy has thus been set up to determine the high-resolution structures of the main functional blocks (33) and of several structural joints, as detailed in Table S1 in Supplementary Material (**Figures 1D–F**). For the C1q molecule, only the X-ray structure of the C-terminal globular domain could be obtained (34), alone or in complex with minimal recognition motifs, such as deoxyribose for DNA, which gave insights into its recognition properties [reviewed previously in Ref. (35)].

More X-ray structures of C1r and C1s protease domains have been determined (Table S1 in Supplementary Material). The structures of all C1s modules are now known (**Figure 1F**). Detailed insights about conformational rearrangements were obtained by comparing different X-ray structures, for example between pro-enzyme and active states of the SP domains (36, 37), as well as some variations in inter-modular orientations (5, 38). The structure of the SP domains also revealed the main structural determinants of their restricted substrate specificity (4, 37, 38). However, C1s SP domain alone is not able to cleave C4 efficiently (39). C4 cleavage, which is the first step of both the classical and lectin activation pathways, appears thus to be more stringent since it requires additional exosites (40). The fine structural details about exosites in MASP-2 (the equivalent of C1s in the lectin pathway) and their interaction with C4 were unraveled recently (41). The functional implication of the homologous CCP exosite in C1s could be confirmed by mutational analyses (41). The structure of the C1s exosite at the CCP1/CCP2 interface was then solved recently (37). Interestingly, both the zymogen structure and surface plasmon resonance interaction analyses suggest that the C1s exosites are partly hidden in the pro-enzyme state (37).

Structural details of protein–protein interactions relevant in terms of C1 assembly were also unraveled during this structural dissection, such as the head-to-tail interaction of the C1r catalytic domains. Such a dimeric interaction has been observed three times by X-ray crystallography and the butterfly-like side view (**Figure 2A**) can also be recognized at the center of early electron micrographs of the proteases tetramer (23, 36, 42). This interaction is maintained through contacts between the CCP1 module of one C1r subunit and the SP domain of its partner (36). One of the functional consequences is the larger than 90 Å distance between the active site of one monomer and the scissile bond of its partner, which prevents spontaneous mutual activation in this dimeric context (36). This auto-inhibited assembly looks like a "resting" state, which requires a conformational change to trigger C1 activation (36, 43). This interface between the catalytic domains of C1r is really specific of the CP activation, with no equivalent in the complexes activating the lectin pathway. Another structural

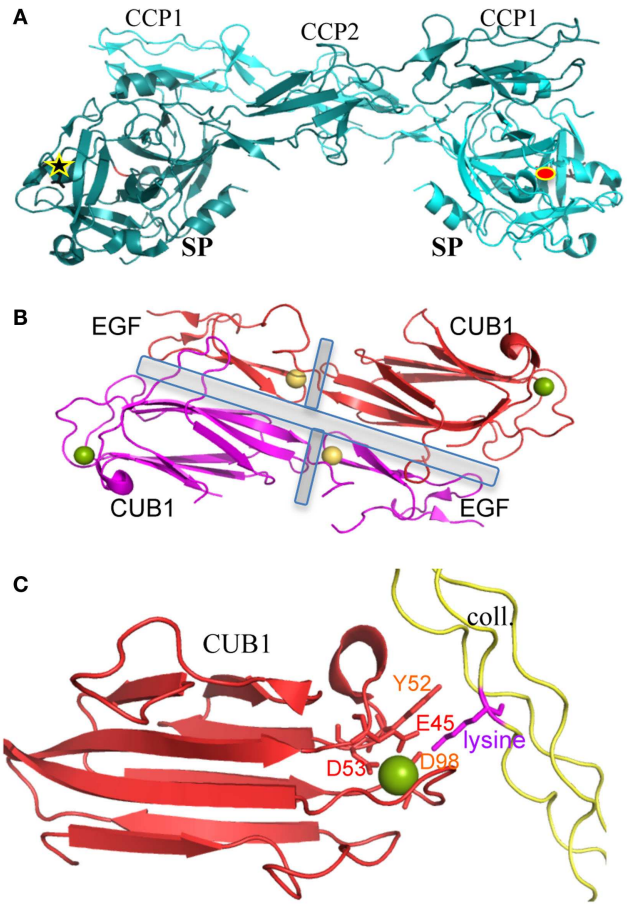

FIGURE 2 | Structures of key protein/protein interactions of C1 components. **(A)** Dimeric association of the C1r catalytic domains. The interface involves similar interactions between the SP domain and the CCP1 module, in both the pro-enzyme and active C1r catalytic domain structures. This typical "butterfly" shape can be recognized on some electron micrographs from the proteases tetramer (23). The 90 Å distant active site (red ellipse) and activation site (black star) of the two molecules are highlighted [PDB code 1GPZ (36)]. **(B)** Dimeric CUB1–EGF interface (present *in vitro* in C1s homodimers and *in vivo* in C1r/C1s heterodimers). The central EGF calcium-binding sites stabilize both the inter- and intra-monomeric CUB-EGF interfaces (highlighted by gray rectangles). Since interface residues are mostly conserved in C1r (compared to C1s), we can assume that this head-to-tail packing observed with C1s homodimers also stands for the C1s–C1r heterodimer. This typical shape can also be recognized on some rare electron micrographs performed on the proteases tetramer (23). Yellow sphere, calcium in EGF; green sphere, calcium or magnesium in the C1s CUB1 module [PDB code 1NZI (45)]. **(C)** Calcium-dependent interaction between C1s CUB1 module and a lysine-containing collagen-like peptide [PDB code 4LOR (5)]. The main structural determinants are highlighted. The lysine side chain directly interacts with Glu45, Asp98, and Ser100. Asp53 is an essential component of the calcium-binding site. Mutations of Glu45, Tyr52, and Asp98 strongly alter C1q-binding properties [reviewed in Ref. (46)].

feature of the C1r zymogen is the inactive occluded conformation of its primary binding site (44).

Calcium-dependent C1 assembly is controlled by the proteases CUB and EGF modules (47). The structural details governing these interactions have been mainly deciphered, although slightly indirectly. The C1r/C1s calcium-dependent interaction is mediated by their CUB1 and EGF modules, which form a head-to-tail dimer under the control of their EGF calcium-binding site (45) (**Figure 2B**). The calcium ion is tightly bound to the C1s EGF module in the context of the CUB1–EGF C1s dimeric interface, since it could not be replaced by lanthanides during soaking experiments used to solve the X-ray structure (45). This head-to-tail interaction can also be recognized on some early electron micrographs of the proteases tetramer (23). Unexpected calcium-binding sites are present in the CUB domains and govern the interactions between the proteases and the C1q collagen-like stems (45, 48). The calcium ion associated to the C1r CUB2 modules appears to be quite labile, although it greatly enhances the structural stability of these modules (49). Site-directed mutagenesis offered a very effective tool to confirm and detail the essential contributions of several amino acids in the full-length molecules: (i) It identified residues essential for C1q binding in C1r: E49, Y56, and D102 in CUB1; D226, H228, Y235, and D273 in CUB2. Other mutations severely affecting the C1q interactions were observed for E45 and Y52 in C1s CUB1 (46, 48). (ii) Conversely, the lysines B61 and C58 in C1q were identified by site-directed mutagenesis as essential protease-binding residues (50). These lysines are very close to the patient mutation GlyB63Ser resulting in a C1q functional deficiency including defective CP activation (12).

Similar CUB and EGF calcium-dependent interactions have then been observed in the MASPs-defense collagens complexes initiating the lectin complement pathway, as well as in other unrelated molecular systems (46, 51, 52). The structure of the C1s CUB1–EGF–CUB2 fragment in complex with a collagen-like fragment containing the OGKLGP sequence (O standing for hydroxyproline, **Figure 2C**) confirmed such a generic mode of association but reveals a different orientation of the CUB2 module as compared to MASP CUB1–EGF–CUB2 fragments (5).

WHAT REMAINS STILL ILL-DEFINED?

Only the C1r CUB modules and the C1q collagen-like domain structures have not yet been solved at atomic resolution, but we know at least their overall shape and scaffold through homology and experimental analyses such as electron microscopy. The structure of the C1q recognition domain where the three subunits (**Figure 1C**) tightly interact with each other in a ACB clock-wise order (as seen from the collagen stem) has also indirectly given some clues about the relative ordering of the three chains in the preceding collagen-like stem (34, 47).

The isolated fragment X-ray structures or models can be combined into hypothetical C1 models (47). These C1-like models illustrate hypotheses in the 3D space about possible modes of C1 assembly and activation, which can then be further tested by site-directed mutagenesis (48). These models are idealized since, for example, C1 is always displayed as a symmetrical molecular complex although we know that it is highly flexible, which disrupts most of its symmetrical conformation in response to the environment. These models also aim to provide a synthetic overall representation consistent with accumulated experimental evidences (7). For example, the model depicted in **Figure 1G** accounted for the differential accessibility of lysine's residues in

C1q and C1 derived from mass spectrometry comparative analyses as well as previous experimental knowledge (7). However, such a dense C1 complex cannot be easily seen on electron microscopy images (unpublished results), and thus the corresponding C1 model remains an "*in silico*" interpretation (as well as most of C1 models).

Part of the "C1 paradox" has thus been elucidated since we know most of the building block structures and also key residues involved in C1 assembly, with now six C1q-binding sites in the protease tetramer (48). Nonetheless, details on how a flexible protease tetramer associates with such a flexible recognition molecule, and how C1 activation proceeds and is controlled remain ill-defined. In contrast to the *in vitro* studies, C1q and C1 can be found *in vivo* under flow conditions, both in the circulation and in the extravascular fluid, where shear stress could affect C1 assembly and activation (53). Moreover, observing fine structural details within C1 still represents a real experimental challenge because of its great flexibility and modular composition. The following questions are thus partially unanswered: How flexible is each inter-modular junction *in vivo*? Is the C1r CUB2 module only partially saturated by calcium *in vivo*, and thus possibly marginally stable within C1 (49)? What is the role of the charged and flexible long insertion in C1r EGF (54)? Which chain is at the leading, medium, and edge position in the native C1q collagen heterotrimeric stem? What are the relative positions between these native C1q stems and the proteases CUB domains? Do the proteases stably stay attached to C1q or is there a fast assembly/disassembly equilibrium? What drives the spectacular conformational change of the proteases from their elongated flexible shape in solution toward the assumed compact C1-associated conformation? How can we observe, describe, or deduce the details of the conformational changes involved during C1 activation? How can we observe the transmission of the triggering signal from C1q recognition to C1r activation? How can we characterize the required C1q conformational change(s)? How is C1r activation propagated to the successive C1s, C4, and C2 activations? How does C1-inhibitor finely control these processes? What about C1 activation by non-immune targets in a physiological or pathological context? How do differences in antigenic structures and surface density precisely modulate the levels of CP activation by the Ab-coated targets? How can we predict the classical C activation outcome when C1q binds to ligands through its globular heads? How do pathogens interfere with C1 activation?

PERSPECTIVES

Over the years, detail after detail, the image describing the immunoglobulins/C1 interaction is gradually emerging. But the flexibility of the C1 molecule and its thin flexible building elements such as the collagen-like stalks make its fine details difficult to observe. Even electron microscopy performed on C1 bound to hexameric IgG surface clusters on liposomes did not fully overcome the limitations due to C1 flexibility, since only four (out of the six expected) globular densities probably corresponding to C1q recognition domains could be consistently observed on top of the hexameric IgG assembly (8). The collagen stems are also too thin, fragile, and flexible to be seen on averaged density maps. Only the position of the larger N-terminal collagen stalk remains visible after averaging. Visible density also remains after averaging for the region probably corresponding to the interaction domains of C1r and C1s, which fill a continuous section inside the C1q cone.

In conclusion, although refining the structural details of C1 assembly and activation remains a difficult challenge, this mission does not sound definitively "impossible." The scientific community will probably find out new solutions to further decrypt the fine structural details, for example by matching X-ray structures and electron density maps obtained from new developments in electron microscopy and associated computing strategies. The use of recombinant C1 fragments (C1q, C1r, C1s) will be useful to further check in detail their structure/function relationships.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00565/abstract

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