C1Q: A MOLECULAR BRIDGE TO INNATE AND ADAPTIVE IMMUNITY

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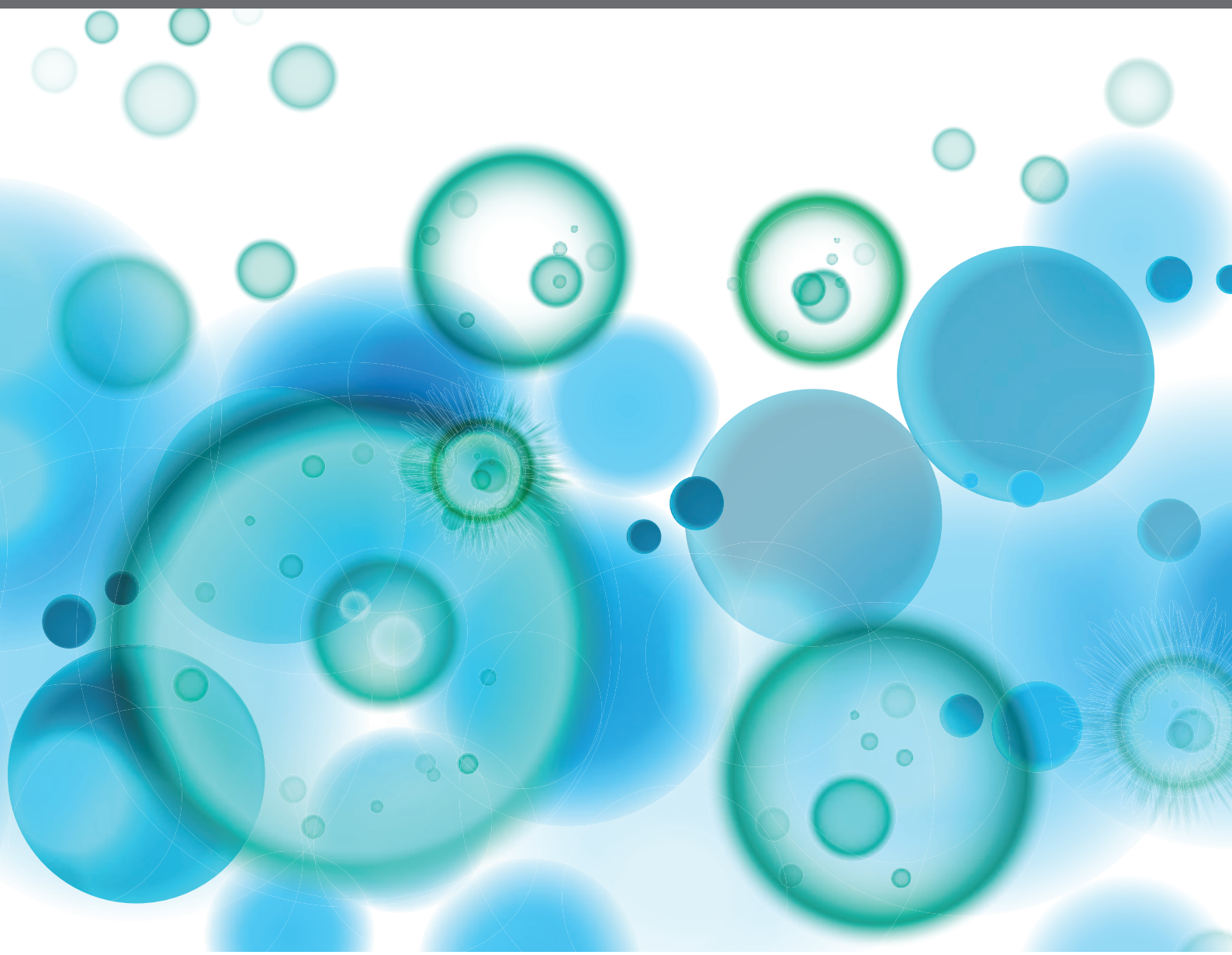

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C1Q: A MOLECULAR BRIDGE TO INNATE AND ADAPTIVE IMMUNITY

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EDITORIAL
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Editorial: C1q: A Molecular Bridge to Innate and Adaptive Immunity

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Editorial on the Research Topic

C1q: A Molecular Bridge to Innate and Adaptive Immunity

Human C1q is the first recognition molecule of the classical pathway of the complement system. C1q has a characteristic tulip-like overall structure where N-terminal collagenous stalk (CLR) is followed by a heterotrimeric C-terminal globular (gC1q) domain (1). After recognizing IgG- and IgM-containing immune complexes, C1q, in association with C1r and C1s complexes that yields C1 molecule, triggers the classical pathway activation. However, C1q is not always dependent on the binding of IgG or IgM to target ligands, primarily pathogens, in order to perform its duty as a potent innate immune molecule. In addition to being the key molecule of the classical pathway, C1q has a broad range of functions that includes clearance of apoptotic and necrotic cells, sustenance of pregnancy, recognition of pathogens in an antibody-independent manner, immune cell modulation, and pruning of excess synapse during development (2, 3). The importance of C1q in human health has been highlighted by its involvement in a number of pathological conditions including lupus nephritis, a number of inflammatory disorders, Alzheimer's disease, prion disease, and cancer.

During the course of last two decades, a number of proteins of diverse origin have been shown to contain at least gC1q-like domain, and hence, identification of a C1q family (4). The structural analysis of one of such C1q family member, adiponectin, revealed that the three dimensional structure of gC1q domain was remarkably conserved and overlapped with tumor necrosis factor (TNF) and related molecules, hence, recognition of a C1q-TNF superfamily (2, 5–7).

Although the main source of the local synthesis of C1q has been attributed to the potent antigen presenting cells such as monocyte/macrophages and dendritic cells, various types of proliferating and non-proliferating cells including malignant cells can also be included in the list of C1q producers (8). Thus, there are a number of functions assigned to C1q that are not reliant on complement activation mediated by C1q (non-complement functions of C1q) (3).

In this volume, which highlights the structural and functional complexities of human C1q, Reid, one of the pioneers in the field, has elegantly provided a historical account of the field, together with many unanswered questions and what the future holds for this truly remarkable complement protein. Another review by Lu and Kishore examines important features of C1 complex that can perform exciting and unexpected functions without involving complement activation (9). For instance, C1q, when bound to the Frizzled receptors, leads to activation of C1s, which cleaves lipoprotein receptor-related protein (LRP) 6 to trigger aging-associated Wnt receptor signaling (10). C1q binds to apoptotic cells and the activated C1 proteases cleave nuclear antigens (11). The diversity of C1q ligands and C1 protease substrates makes C1q as well as C1 complex quite a versatile recognition and effector machinery beyond the territory of complement activation. In the continuing theme of structural and functional versatility of C1q, Ghebrehiwet et al. emphasize the modular organization of the gC1q domain, revealing the 'secret of this functional diversity',

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based on the modularity within each chain of gC1q domain i.e., ghA, ghB, and ghC modules (12). Within the gC1q domain, which is composed of the C-terminal ends of A, B and C chains, this review makes arguments for the A-chain (ghA) to be most versatile module in terms of ligand binding (11).

C1q binding to the CH_2 domain of antigen-bound IgG and subsequent classical pathway activation depends on its close proximity to the Fc region of adjacent IgG; C1q does not bind (or binds with very weak affinity to) circulating IgG monomers. Crucially, IgG CH_2 domains contain Asp-297 N-linked glycan amenable to extension by terminal galactose and sialic acid residues. Using recombinant variants of CD20-specific monoclonal antibody, rituximab, Perschke et al. demonstrate that Fc-galactose enhances complement fixation, but only for IgG1 and IgG4, proposing a novel strategy to manipulate complement fixing ability of therapeutic antibodies. Following up on the C1q-IgG subclass interaction, Lilienthal et al. have examined the parallel between murine IgG1 and human IgG4 subclasses (both capable of inhibiting hexamerisation of IgG1 and IgG3 and subsequent C1q binding and classical pathway activation). The authors show that murine IgG1 suppresses IgG2a-mediated classical pathway activation. Since IgG subclass is of great importance in pathophysiology, galactose and sialic acid manipulation has therapeutic implications (Lilienthal et al.). It is worthwhile to note that allergen-based immunotherapy in allergic patients can give rise to increased IgG4 levels while dampening specific IgE production; thus, IgG4 subclass polarization also circumvents the classical pathways activation in allergen-desensitized patients (13). Moving on from immunoglobulin interaction of C1q to non-immune ligands, Donat et al. examined the implications of C1q binding to cholesterol crystals as well as von Willebrand factor (vWF) with respect to cholesterol crystal clearance by macrophages. Curiously, vWF bound cholesterol crystals via C1q, and the tripartite complex upregulated phagocytic and sensing receptors, such as MerTK, LRP-1, SR-A1, CD14, LAIR1, and PD-L1. vWF seems to interfere with the phagocytosis of cholesterol crystals and C1q complex. An assessment of pro-inflammatory cytokines revealed that vWF binding to C1q suppresses inflammatory response by macrophages, which may be relevant in atherosclerosis. El-Shamy et al. have reviewed the role of complement (and C1q) in chronic hepatitis C virus (HCV) infection and cryoglobulinemia since HCV-triggered complement activation is involved in liver fibrosis and type II cryoglobulinemia.

The last two papers, from Roberta Bulla group, in the volume allude to the fascinating involvement of C1q in tumor (Agostinis et al.; Mangogna et al.). C1q is expressed in the microenvironment of various types of human tumors, including melanoma, prostate, mesothelioma, and ovarian cancers. C1q promotes tumor by encouraging their adhesion, migration, and proliferation in addition to angiogenesis and metastasis (14). Agostinis et al. now report that C1q is found in good amounts in the tumor microenvironment of asbestos-induced malignant pleural mesothelioma (MPM), where it can interact with hyaluronic acid (HA), an abundant tumor microenvironment component. C1q-HA interaction seems to work in favor of MPM cells, suggesting that C1q can be exploited by tumor for its progression and invasion. In another study, Mangogna et al. have used Oncomine and UALCAN database in order to ascertain whether the transcriptional expression of the C1q three chains has a prognostic relevance for glioma. C1q is known to be expressed in the central nervous system and is considered a precipitation factor for neurodegeneration and neuroinflammation (9). The study reveals a positive correlation between higher levels of C1q expression and unfavorable prognosis in a diverse grade of gliomas, thus, giving a new dimension to C1q research. How C1q interacts with brain tumor cells as well as microglia and astrocytes in the context of gliomas needs further investigation.

In conclusion, C1q remains an ever important molecule of complement and innate immunity. Its versatility and modularity seem to offer enormous physiological potential; sometimes, it can be exploited by pathogens and tumor to their end. Future research in the area of cancer, pregnancy, aging and neuroinflammation is going to throw many pleasant surprises.

AUTHOR CONTRIBUTIONS

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

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Complement Component C1q: Historical Perspective of a Functionally Versatile, and Structurally Unusual, Serum Protein

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Complement component C1q plays an important recognition role in adaptive, and innate, immunity through its ability to interact, *via* its six globular head regions, with both immunoglobulin and non-immunoglobulin activators of the complement system, and also in the clearance of cell debris, and by playing a role in regulation of cellular events by interacting with a wide range of cell surface molecules. The presence of collagen-like triple-helical structures within C1q appears crucial to the presentation, and multivalent binding, of the globular heads of C1q to targets, and also to its association with the proenzyme complex of $C1r_2$–$C1s_2$, to yield the C1 complex. The possible role that movement of these collagen-like structures may play in the activation of the C1 complex is a controversial area, with there still being no definitive answer as to how the first C1r proenzyme molecule becomes activated within the C1 complex, thus allowing it to activate proenzyme C1s, and initiate and the consequent cascade of events in the activation of the classical pathway of complement. The globular heads of C1q are similar to domains found within the tumor necrosis factor (TNF) superfamily of proteins, and have been shown to bind to a very wide range of ligands. In addition to its well-defined roles in infection and immunity, a variety of other functions associated with C1q include possible roles, in the development of problems in the central nervous system, which occur with aging, and perhaps in the regulation of tumor growth.

Keywords: C1 activation, C1q, collagen-like structure, globular heads, tumor, aging

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INTRODUCTION

Prior to the formal proof of there being collagen triple helical coils present in the C1q molecule (1), it was considered that any such protein, containing that feature, would most likely play a structural role in the extracellular matrix, rather than being involved in the activation of the serum complement system. However, it was recognized by the end of the 1980s (2) that several other serum proteins, besides subcomponent C1q, also contained collagen-like regions, and were likely to be involved in innate immune effector systems. These included the C-type lectins, mannose binding lectin (MBL), bovine conglutinin, and lung surfactant protein A. Indeed, conglutinin was the first vertebrate lectin to be characterized by virtue of its function to promote the agglutination of

Abbreviations: CTRP, C1q/TNF-related protein; MBL, mannose binding lectin; MASP, MBL-associated serine protease; CRP, C-reactive protein; CUB, complement C1r/C1s; EGF, epidermal growth factor.

erythrocytes coated with activated complement components (3). It is now known that there are several other lectins, such as lung surfactant protein D, the ficolins, bovine serum lectin (CL-43), in a growing family of proteins, containing collagen-like regions, which are involved in immune defense. These proteins display a wide range of binding properties, *via* both their globular head regions and collagen-like triple-helical regions, toward both immune targets and cell surface receptors, and participate as a bridge between innate and adaptive immunity. The relationship between structure and function with respect to the many binding, and triggering, properties shown by C1q should now be able to be even more fully explored, by generation of point mutation variants, as a result of the major achievement of expression of the functionally fully active recombinant form of this structurally complicated protein, composed of three different polypeptide chains, in a mammalian cell system (4).

EARLY CHARACTERISATION OF C1q AND DETERMINATION OF ITS STRUCTURE

The C1q protein was first, accurately, described, in 1961, as a "11s thermolabile serum protein which precipitates γ-globulin aggregates and participates in immune hemolysis" (5), thus highlighting interesting features about its large size (460 kDa) and its binding properties (to immunoglobulin complexes) and its function (participation in complement-mediated hemolysis of antibody-coated red cells). In 1963 (6), Lepow et al. showed that the euglobulin fraction of human serum (proteins precipitated in low ionic strength buffer, at pH 5.5), which contained the then defined C1component of the complement system, could be fractionated, by ion-exchange chromatography into three subcomponents, which were defined, based on their elution positions, from an ion-exchange column, as C1q, C1r, and C1s (the nomenclature a, b, and c…, was not used, in order to avoid confusion with "C1a" being used for activated C1, at that time). It was shown that all three subcomponents were required to reconstitute the original C1 hemolytic activity. The use of further, new at the time, techniques, such as gel-filtration and affinity chromatography, allowed the isolation of highly purified C1q to perform detailed structural and functional studies.

Early chemical studies of human and rabbit C1q (7–9) provided indirect evidence, that there may be collagen-like structures within C1q, since it was reported to have an unusually high glycine content, to contain hydroxylysine and hydroxyproline residues and disaccharide units of glucosylgalactose, linked to the hyroxylysine, and it had a great susceptibility to collagenase. The first direct evidence for the presence of collagen-like amino acid sequence, in the A-chain of C1q, was obtained in 1974 (10).

It was then shown that the preparation of the collagen-like regions of C1q could be achieved by limited proteolysis of the native intact molecule with pepsin at pH 4.45, when the globular head regions are digested to small peptides leaving the large, 190 kDa, collagen-like region intact (1, 11). When viewed in the electron microscope, C1q was seen to be composed of six peripheral globular "head" regions, which are each joined by a collagen-like connecting strand to a fibril-like central portion/stalk (1, 12, 13).

All these studies allowed the proposal in 1976 (14) of a molecular model for subcomponent C1q (**Figure 1**), which has stood the test of time, in which there are 18 polypeptide chains (6 A-, 6 B-, and 6 C-chains), with disulfide bonds between the A- and B- chains and between pairs of C-chains, thus yielding nine dimers, i.e., six A–B dimers and three C–C dimers. The complete derived amino acid sequence, along with the characterization and organization of the genes encoding all three polypeptide chains of C1q, was completed in 1991 (15).

The use of large fragments of C1q, produced by limited proteolysis with either collagenase or pepsin, allowed direct analysis of the primary functions of the two, very different, regions of the C1q molecule. The globular head regions of C1q can be prepared by digestion with collagenase at pH 7.4, which results in a rapid loss of C1q function (9, 16). These preparations, of globular subunits, with a molecular weight of 47 kDa, were shown (16) to be able to inhibit the binding of C1q to IgG immune complexes, thus directly illustrating the role of the peripheral globular head units in binding to IgG. The determination of the crystal structure globular head region of C1q (17), also prepared by collagenase digestion, has shown that it is an almost spherical heterotrimeric assembly (formed from the C-terminals regions of the A-, B-, and C-chains). This allowed molecular modeling with two of its well-defined targets, IgG and C-reactive protein (CRP), providing a good illustration of the versatility in binding shown by the globular heads of C1q. It was found that the large pepsin-resistant fragment of C1q, composed almost entirely of the collagen-like regions of the molecule, could act as an effective inhibitor of the reconstitution of whole C1 hemolytic activity, when intact C1q was mixed with $C1r_2$–$C1s_2$ (18). It has also been found that the pepsin-resistant fragment of C1q bound the unactivated $C1r_2$–$C1s_2$ with approximately the same strength as that of the intact C1q molecule (19). These studies provided strong functional evidence that the $C1r_2$–$C1s_2$ interacted primarily with the collagen-like regions of C1q, and was in agreement with electron microscopy studies of the whole C1 complex (20) which suggested that the $C1r_2$–$C1s_2$ binding site is in the middle of the triple helical collagen-like regions, close to where the collagen-like strands diverge out from the central fibril-like region (**Figure 1**).

STRUCTURAL MODEL OF THE COLLAGEN-LIKE REGION OF C1q, ITS INTERACTION WITH $C1r_2$–$C1s_2$ AND POSSIBLE INVOLVEMENT IN ACTIVATION OF THE C1 COMPLEX

It is clear from electron microscopy, and all the protein structural data available, that the 460 kDa C1q molecule adopts a bouquet of flowers shape, comprising six heterotrimeric collagen-like triple helices that associate in their N-terminal half to form a fibril-like structure, then diverge at a bend, or "kink," approximately half-way along the collagen-like region to form six individual "stalks," each terminating in a C-terminal

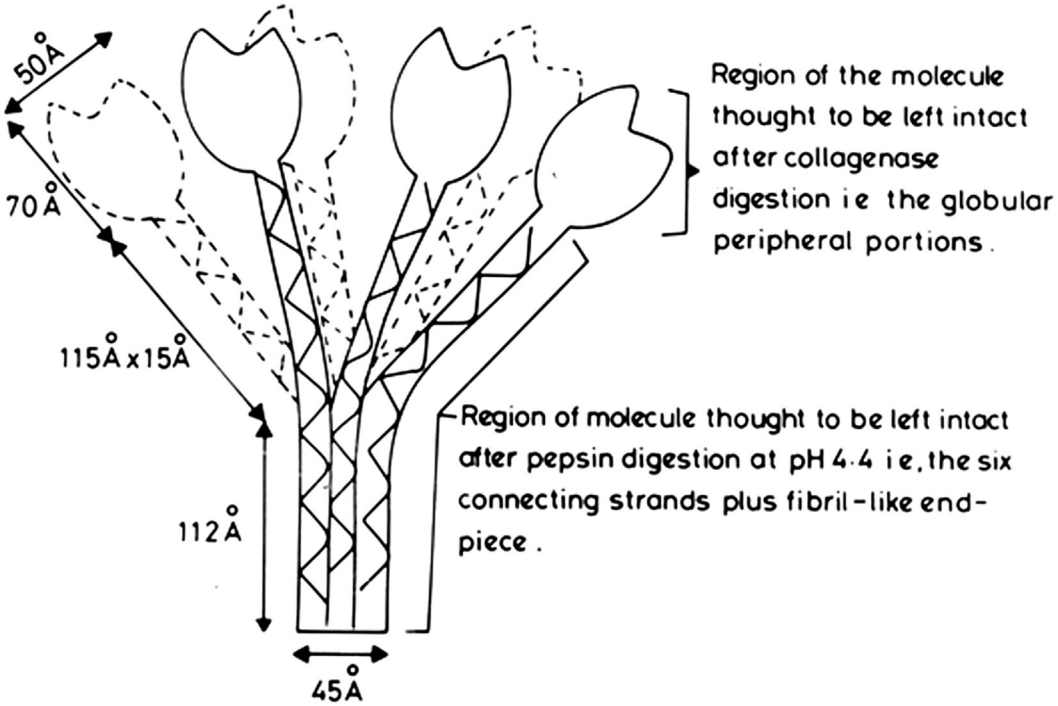

FIGURE 1 | Proposed model of human subcomponent C1q. Initial diagram, drawn up in 1975, of the first published molecular model proposed for C1q (14). It was based on the electron microscopy measurements (12, 13), the amino acid sequencing and physical chemistry results of the studies on the 190 kDa pepsin-resistant fragment of C1q (1, 11), and the assumption that the collagen-like regions in the A-, B-, and C-chains of C1q form a triple helical collagen-type structure (denoted by the solid, broken, and wavy lines), and the C-terminal approximately 140 amino acid residues, in each of the A-, B-, and C-chains, form a globular heterotrimeric structure of 47.8 kDa (which should, more correctly, be shown as globular units, rather than the tulip-flower shapes as shown in the initial diagram). From the dimensions shown, which are averages of those given in the electron microscopy studies (12), the following comparisons can be made: length of collagen-like fiber plus fibril-like end piece = 115 + 112 = 227 Å. Length of triple helix proposed from amino acid sequences = 78 × 2.9 = 226 Å (2.9 Å is the rise of collagen helix per residue, and there are 78 residues, over the collagen-like regions, in each of the three chains of C1q).

heterotrimeric globular domain (**Figure 1**). Other serum proteins (MBLs, Ficolins) have an overall structural similarity to C1q, and also interact with their associated proteases *via* their collagen-like regions. It was noted that, on alignment of all the known, collagen-like sequences, present in various chains of C1q, MBL, and Ficolin, from several species, that there is a conserved amino acid sequence (-Hyp-Gly-Lys-Xaa-Gly-Pro-) in which it was shown, by site-directed mutagenesis of Ficolin A, that the lysine is the critical residue involved in interaction with its associated protease, MASP (21). This conserved site, also present in each of the three chains of C1q, is six Gly-Xaa-Yaa- triplets C-terminal to the link region of C1q, and it was, therefore, postulated that this is likely to be the major binding site for $C1r_2$–$C1s_2$ on the stalks of C1q (21). The importance of these lysine residues, in the B- and C- chains, and lesser extent the A-chain, for the interaction, and activation, of $C1r_2$–$C1s_2$ was elegantly formally proved by their mutation to alanine residues, with consequent loss-of-function. Further studies, involving the determination of the crystal structure of the CUB1-EGF-CUB2 region of C1s bound to a short triple helical collagen-like peptide, containing the important conserved lysine, provided strong direct structural evidence for the proposed, precise interaction site between C1q and $C1r_2$–$C1s_2$ (22).

C1q TARGETS AND RECEPTORS

The structural studies on globular head region of C1q show that it is member of the growing TNF superfamily (17, 23) and functional studies indicate that the C1q heads can bind a wide range of self and non-self ligands.

Primary targets for the six globular heads of C1q are the multiple Fc regions presented within immune complexes, containing IgG or IgM antibodies. Well-defined non-immunoglobulin targets, for the C1q heads, include CRP (24) and pentraxin 3, as well as lipopolysaccharides and bacterial porins. Apoptotic cells also form a major target (25–28), probably *via* C1q binding to phosphatidylserine and double-stranded DNA, thus allowing for opsonization and effective phagocytosis of cell debris (28, 29), and enhancement of the immunosuppressive nature of the apoptotic cells.

One of the first molecules to be proposed as a receptor, for the globular heads of C1q, is gC1qR/p33 (30), but this molecule, although found at the surface of a wide variety of cells, has been shown to be present mainly in mitochondria. Since it does not possess a transmembrane domain, or a lipid anchor, it appears that it must always have to interact with other cell surface molecules, in order to modulate intracellular functions.

After activation of the C1 complex, control of the activated C1r and C1s is mediated by C1-inhibitor which forms covalent complexes with both the activated C1r and C1s, rapidly removing them from the C1 complex, leaving the entire collagen-like region of C1q free to interact with potential cell-surface receptors. One such putative receptor was a 60 kDa molecule isolated from Raji cell membranes (31) and to bind to the collagen region of C1q (and thus became to be defined as cC1qR). The NH2-terminal amino acid sequence of this molecule, isolated from endothelial cells (32) was found to be identical to that of C1qR, isolated from tonsil lymphocytes, and also to that of calreticulin (33) The C1qR preparation was shown to bind to the collagen-like regions of C1q and several other collagen-like proteins (34). Although cC1qR/C1qR/calreticulin can be found at the surfaces of many cell types, it is primarily found in the endoplasmic reticulum, and lacks a transmembrane domain. Thus care must be taken to define both gC1qR and cC1qR as "C1q-binding proteins," rather than as true "transmembrane receptors."

A wide range of other cell surface molecules have now been shown to interact with the either the heads, or collagen-like regions, of C1q, and occasionally with both. This area has recently been thoroughly and critically reviewed (35). The putative receptors were neatly placed in four main groups, based on the nature of their extracellular domains: (i) large multi-modular ectodomains involved in interaction with multiple ligands (CR1, LRP1, and the scavenger receptors SR-F1 and SR-F3); (ii) integrins (α2-β1 and CR3/αM-β2); (iii) Ig-like receptors (RAGE, LAIR-1, and CD33); and (iv) the C-type lectin receptors, DC-SIGN and DC-SIGNR.

CURRENT VIEWS AS TO THE MECHANISM OF ACTIVATION OF THE C1 COMPLEX

There are two recent publications (36, 37), which address the possible mechanism by which it is considered the first steps of activation of proenzyme C1r, within the C1 complex, may take place, and they come to significantly different conclusions.

One view, the intramolecular view (36), is that the proenzyme C1r domains must be linked together at the center of the hetero-tetramer $C1r_2$–$C1s_2$, and that autoactivation of proenzyme C1r occurs as soon as the contacts between the catalytic domains are broken, possibly by flexibility of the collagen-like stems of C1q, on contact of the globular heads with targets. These conclusions derive from previous observations and are associated with studies on the interactions, in solution, of fragments (the CUB1-EGF-CUB2 fragments) of the C1r and C1s involved in binding to C1q, and also the crystal structure of a complex of these two fragments, allowing a close analysis of the C1s–C1r interface.

The other view, the intermolecular view (37), is that the C1 complex cross-activates by interacting with neighboring C1 complexes. This model is based on synchrotron small-angle X-ray scattering and electron microscopy studies and concludes that there is cleavage of proenzyme C1r in one C1 complex by C1r in a neighboring complex. The two models differ in the precise manner in which the $C1r_2$–$C1s_2$ complex is aligned, at the now generally agreed position, within the collagenous stems of C1q. The intramolecular view (36) does appear to be consistent with the first-order kinetics, reported by several groups, as regards activation of C1, but does rely upon contacts between the catalytic domains being broken, presumably by flexibility within the collagen-like stems on binding to a target, and this has not yet been formally proven. However, although collagen-like structures are seen as structures with only limited flexibility, all observations, made on C1q, suggest flexible hinge movements at the level of the "kink," which can modulate the positions of the six globular domains. The intermolecular model (37), in which the serine protease domains are considered to be located at the periphery of the $C1r_2$–$C1s_2$ complex is consistent with the fact that C1 can bind to a structurally diverse range of activators and allows intermolecular activation between neighboring complexes. In a very recent study (38), cryo-electron microscopy was used to examine C1 bound to monoclonal antibodies, and the authors observed heterogeneous structures of single and clustered C1-IgG1 hexamer complexes. This structural data was interpreted as showing that, upon antibody binding, the C1q arms condense, thus inducing rearrangements of the $C1r_2$–$C1s_2$ complex and tilting the C1q's cone-shaped stalk. Thus, it was concluded that C1r perhaps could activate C1s within single, strained C1 complexes, or between neighboring C1 complexes on surfaces (38).

Final general acceptance of one, or other, or indeed a combination, of these models of C1 activation awaits further study.

BIOSYNTHESIS OF C1q, LEVELS OF SERUM C1q, AND DEFICIENCY OF C1q

Unlike most of the other complement proteins, which are mainly liver-derived, C1q is synthesized primarily by macrophages, as demonstrated by the fact that bone marrow transplantation from wild-type mice into C1q-deficient ($C1qa^{-/-}$) mice was able to restore the normal serum levels of C1q (39). This finding has prompted the use of hematopoietic stem cell transplantation in the remarkable, and successful, treatment of genetic human C1q deficiency (40), a condition where the complete absence of C1q function results in an exceptionally high risk of severe lupus erythematosus, and complications with skin and renal diseases.

In normal healthy human sera, C1q has a concentration of around 80 μg/ml (0.17 μM in serum) and thus is present in an approximately equimolar concentration to that of the $C1r_2$–$C1s_2$ complex (50 μg/ml of each of C1r and C1s, thus 0.15 μM $C1r_2$–$C1s_2$). The concentration of C1q rises quite steeply with aging, reaching 161 μg/ml, in the 60–81, years old, age group (41). It is of interest that the high serum level of C1q could surprisingly be reduced by a simple exercise regime, down to almost normal levels, which may be of significance to health in old age. An even more dramatic (10- to 300-fold) increase in C1q levels in the central nervous system (42) has been reported in mouse and human brains, with the highest levels being seen in close proximity to synapses and central regions of the brain. Interestingly, aged

C1q-deficient ($C1qa^{-/-}$) mice showed less cognitive and memory decline in hippocampus-dependent behavior tests compared to their wild-type litter mates (42), thus suggesting that C1q may play a role in the development of problems, during aging, that are seen in the central nervous system.

Another connection between C1q and aging has emerged from studies that there is elevated Wnt signaling in aging mice, where muscle stem cells have an increased tendency to fibroblastic differentiation (43). This appears to be due to the binding by one, or more, serum factors, to the Frizzled family of cell surface receptors, thus causing Wnt receptor signaling, and one Frizzled-binding protein has been identified to be C1q (44). It has been proposed that when C1q, within the C1 complex, binds to Frizzled then activated C1s cleaves lipoprotein receptor 6, which is a Wnt co-receptor, thus causing canonical Wnt signaling and accelerated aging. Thus abnormally high, or low, C1q levels could possibly play a role in various disease states caused by increased, or decreased, Wnt signaling.

There is growing evidence that C1q binding, leading to activation of C1r and C1s, may trigger other functions *via* activated C1s, unexpectedly, cleaving nuclear antigens, MHC class I antigens and other proteins, as well as lipoprotein receptor 6 (45), thus opening up many other biological pathways, besides complement, that may be triggered by C1q binding.

A recent study has indicated that non-bone marrow derived, locally synthesized, C1q may play a role in enhancing tumor progression by facilitating cancer cell seeding and promoting angiogenesis (46). This view is consistent with the finding of increased expression of the genes of the chains of C1q correlating with a poor prognosis in breast cancers (47). However, other studies indicate that C1q may possibly play a protective role, as judged by C1q-enhanced apoptosis in an ovarian cancer cell line (48) and activation of a tumor suppressor to induce apoptosis in prostate cancer cells (49).

CONCLUSION

There have been many publications concerned with the possible functions mediated *via* C1q, since its first description in 1961 (5), and perhaps more the use of the well-defined, globular, and collagen-like, fragments of C1q, monoclonal antibodies against these regions, or mutation of specific residues within the expressed whole recombinant molecule (4), will aid understanding of how exactly C1q is interacting with the large number of targets (in the region of 100, to date) to which it has been reported to bind. The use of a short synthetic triple helical peptide, corresponding to the binding site on C1q for the $C1r_2$–$C1s_2$ complex (22), to define the precise region on C1q involved in that interaction shows there are possibly many other innovative ways, utilizing protein fragments or synthetic peptides, which will allow the exploration, at the molecular level, of the functions of this versatile molecule.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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C1 Complex: An Adaptable Proteolytic Module for Complement and Non-Complement Functions

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Complement C1 is the defining component of the classical pathway. Within the $C1qC1r_2C1s_2$ complex, C1q functions as a molecular scaffold for $C1r_2C1s_2$ and C1q binding to its ligands activates these two serine proteases. The classic C1q ligands are antigen-bound antibodies and activated C1s cleaves C4 and C2 to initiate the complement cascade. Recent studies suggest broad C1 functions beyond the complement system. C1q binds to the Frizzled receptors to activate C1s, which cleaves lipoprotein receptor-related protein 6 to trigger aging-associated Wnt receptor signaling. C1q binds to apoptotic cells and the activated C1 proteases cleave nuclear antigens. C1s also cleaves MHC class I molecule and potentially numerous other proteins. The diversity of C1q ligands and C1 protease substrates renders C1 complex versatile and modular so that it can adapt to multiple molecular and cellular processes besides the complement system.

Keywords: complement C1, autoimmunity, aging, infection, inflammation, C1q, macrophage, dendritic cell

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INTRODUCTION

In invertebrates, complement takes primitive forms represented only by a few ancestral proteins and lacks the specificity and sophisticated regulatory mechanisms of the modern vertebrate complement system (1–4). In mammals and other higher vertebrates, the complement system is a complex protein network consisting of nearly 30 plasma proteins. Depending on the target ligands, the complement system can be activated *via* the classical, alternative, or lectin pathway (5, 6). In the case of microbial pathogens, each complement pathway is triggered through a specific mechanism of ligand recognition, and collectively, the three pathways empower this humoral system to defend against a broad range of microorganisms. Like the blood coagulation system, the complement system is orchestrated around serine proteases, which are sequentially activated and then cleave specific downstream complement proteins so as to amplify a cascade of reactions (2, 7, 8). These reactions generate proteolytic or lytic complexes, opsonins, and peptide anaphylatoxins leading to lysis, inflammation, and clearance of opsonized microorganisms (**Figure 1**) (5, 6). The complement serine proteases exhibit conserved active sites (2). However, these proteases are highly specific for substrate within the complement network, and this appears vital for the directional amplification of each pathway.

Abbreviations: IGFBP5, insulin-like growth factor-binding protein 5; MBL, mannose-binding lectin; MASP, MBL-associated serine protease; SLE, systemic lupus erythematosus; LRP6, low-density lipoprotein receptor-related protein 6; CRP, C-reactive protein; DC, dendritic cell; NPM1, nucleophosmin-1; CTRP, C1q/TNF-related protein.

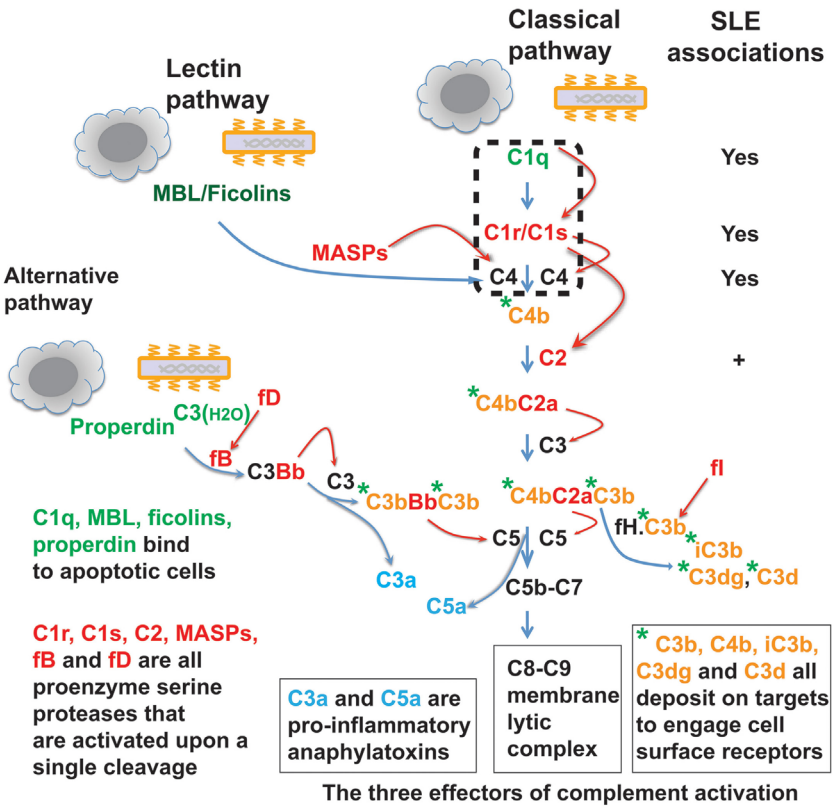

FIGURE 1 | **The complement protein and protease network**. The complement system operates *via* three target recognition pathways: the classical, alternative, and lectin pathways. All pathways recognize microorganisms and apoptotic cells and the recognition subcomponents are in green. Upon its triggering *via* any of the three pathways, the complement acts through three effector reactions: the C8-C9 lytic or membrane attack complex, the soluble C3a and C5a anaphylatoxins (blue color), and surface-bound C3b, C4b, and further proteolytic fragment opsonins (orange color with green asterisk). All three pathways converge at the C3 component and complement reactions are essentially amplified through cascades of serine proteases (red color). MBL, mannose-binding lectin; MASPs, MBL-associated serine proteases; fB, factor B; fD, factor D; fI, factor I; fH, factor H. Homozygous deficiency of C1q, C1r/C1s, or C4 is causally associated with systemic lupus erythematosus (SLE) pathogenesis. Genetic C2 deficiency also increases risk for SLE and some other autoimmune diseases.

The complement system is commonly intended for host defense against microbial infections. Recent data suggest that various non-microbial exogenous and endogenous structures, such as apoptotic cells, may also trigger the complement pathways (**Figure 1**) (9–15). The effects of complement activation may also be delivered through a segment of the system rather than in its entirety. For example, the C1s protease apparently cleaves non-complement proteins including MHC class I molecule, insulin-like growth factor binding protein 5 (IGFBP5), Wnt receptor, and nuclear autoantigens (16–21). This suggests that, besides its well-defined roles in host defense, the C1 complex functions broadly, e.g., in tissue homeostasis and immune tolerance. In fact, invertebrates also utilize their limited repertoire of complement components to clear damaged cells as well as invading microorganisms (22, 23).

THE CLASSICAL PATHWAY IS A MODERN PATHWAY

During evolution from invertebrates leading up to higher vertebrates, animals experienced major genomic expansion through gene duplication and recombination, with higher vertebrates acquiring increased complexity in genomic composition, body plans, and physiological processes (24). The expansion of the complement system in higher vertebrates includes at least two aspects: the generation of paralogous complement elements and the formation of a new classical pathway. In invertebrates, ancestral complement elements were only found that were equivalent to the alternative and lectin pathways, including ancestral C3, factor B, mannose-binding lectin (MBL), ficolins, and MBL-associated serine proteases (MASPs) (3, 22). The modern C1 complex, i.e., the $C1qC1r_2C1s_2$ pentamer that defines the recognition component of the classical pathway, only appeared from jawed vertebrates when adaptive immunity also emerged.

Complement gene duplication and recombination are evident in higher vertebrates, e.g., factor B/C2, C3/C4/C5, and C6/C7/C8/C9 (3). Evidence that the C1r and C1s genes are relatively modern duplications is also suggested by their close genomic proximity and structural similarity (8, 25). This is even more striking with the three C1q subunit genes, i.e., C1qA, C1qB, and C1qC, which are clustered within a 30-kb genomic region separated by short intergenic sequences (26, 27) (**Figure 2**). The closest C1q-related protein in invertebrates is encoded by a single gene and the C1q-like protein recognizes carbohydrates rather

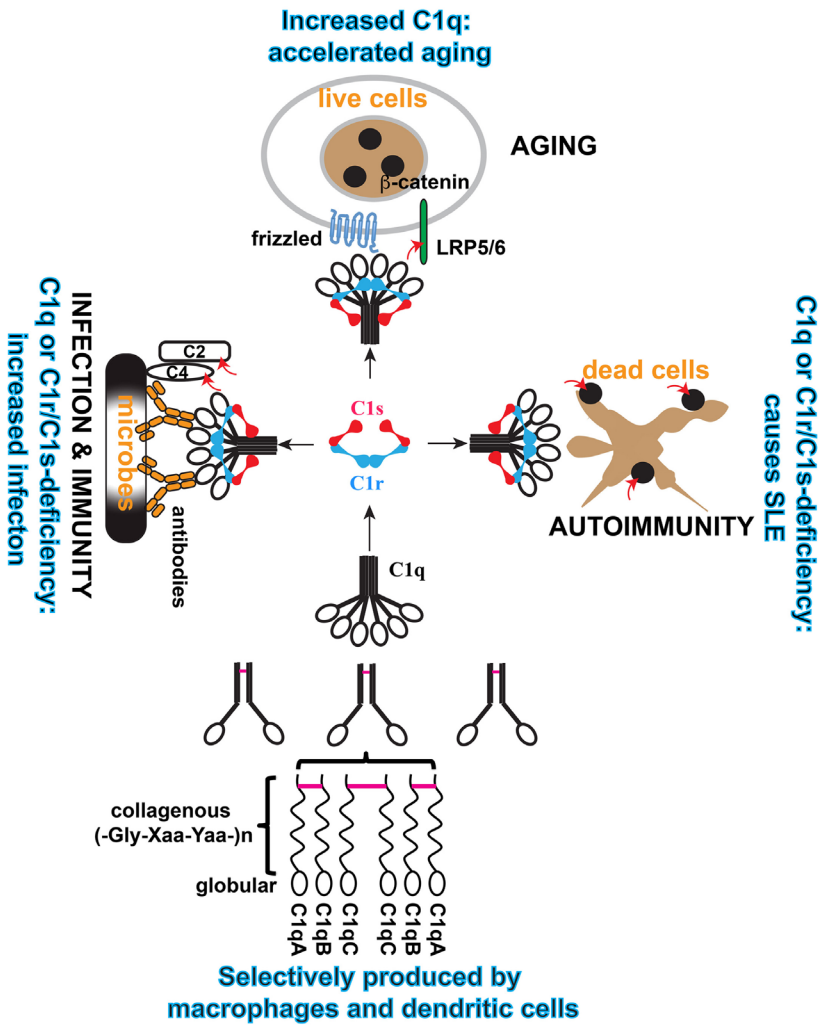

FIGURE 2 | Schematic illustration of the cellular origin of C1q, its assembly, and three distinct modular functions of C1 complex. C1q is an abundant plasma protein. It can be produced broadly in tissues by macrophages and DCs, which also produce C1r/C1s. C1q is formed from three similar but distinct subunits, i.e., A, B, and C, and the order of assembly is illustrated. Inter-subunit disulfide bonds (purple bars) and the collagen-like helices defines the sorting of the three subunits within the C1q polypeptide. In the serum, C1q is associated with the serine protease proenzyme tetramer $C1r_2C1s_2$ to form the C1 complex. In this complex, C1q binds to diverse targets that activate the serine proteases and the proteases trigger effector reactions by cleaving specific substrate. Three physiological contexts are highlighted in which the C1 complex is known to play a role. When C1q binds to antibodies on microbial pathogens, the activated C1s cleaves complement C4 and C2 to initiate the proteolytic cascade. When C1q binds to Frizzled, activated C1s cleaves lipoprotein receptor-related protein 6 to cause canonical Wnt signaling and accelerated aging. When C1q binds to apoptotic cells, the activated C1s cleaves apoptotic cellular antigens to reduce autoimmunogenicity. The red arrows indicate C1s cleavage of the specific substrate.

than immunoglobulins (4). The emergence of the C1 complex or the classical pathway in higher vertebrates, which coincided with the appearance of the adaptive immune system, makes it a "modern" arm of the complement system that responds to antibodies and other self, non-self, and altered self targets.

C1q DEFICIENCY IS A STRONG CAUSE OF SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) PATHOGENESIS

Genetic deficiency has been identified for many complement proteins and, in most cases, this increases susceptibility to infections (28, 29). Deficiency in some complement proteins is also associated with other pathological conditions and particularly strong associations were found between deficiencies in the early components of the complement classical pathway and the autoimmune disease SLE (29–34). The association is especially strong with homozygous C1 and C4 deficiencies. Functionally, C1q binding to ligands causes C1r and then C1s activation and the activated C1s cleaves C4 and then C2 to initiate the further downstream complement cascade (5, 6). C2 deficiency is more prevalent than C1 and C4 deficiencies, but it has substantially less effect and is also associated with other autoimmune diseases (31, 32). However, C1q, C1r/C1s, and C4 deficiencies cause predominantly SLE-like conditions.

In C1 and C4 deficiencies, the disease manifestations also deviate from that found in the larger SLE patient population. Typically, this specific group of SLE patients exhibit early disease onset and equal disease risks from both genders (30, 31, 35). SLE is otherwise a chronic disease that affects predominantly females at childbearing ages (36). How deficiency in each of these intimately related complement proteins, which define the classical pathway (**Figure 1**), causes SLE remains incompletely understood.

SYSTEMIC LUPUS ERYTHEMATOSUS

Clinical documentation of SLE disease has existed for more than a century. In 1948, Hargraves pioneered the mechanistic investigation of this disease by reporting the L.E. cell phenomenon, i.e., SLE patient serum caused polymorphnuclear leukocytes to bind or clump around autologous amorphous nuclear materials (37). The serum activity was later attributed to the γ-globulin fraction of the patient serum, presently known as autoantibodies reactive with chromatin or DNA (38–40). A pathogenic role for these autoantibodies became apparent when Tan et al. reported the asymptomatic appearance of anti-DNA autoantibodies, which disappeared during the ensuing disease flare when serum DNA antigen surged to complex with these autoantibodies (41). These autoantibodies are hallmarks in SLE pathogenesis and deposit in tissues leading to inflammatory tissue injury (42–44).

For a large majority of SLE patients, there is no definitive genetic explanation for the disease despite more than 50 SLE risk genes that have been identified (45). Most of these susceptibility genes are not specific for SLE and individually each risk gene has low-to-moderate effect on the disease (32). Known exceptions are genetic deficiencies of the intracellular exonuclease Trex1, and complement C1 and C4 (30–32). How deficiency in each of these complement proteins overrides the complex mechanisms governing host immunity and tolerance to cause this complex autoimmune disease is not fully understood. As anti-nuclear autoantibodies are pathogenic in SLE, understanding how these deficiencies cause anti-nuclear autoimmunity can provide greater insights into the underlying pathogenic mechanisms.

PLASMA C1q ACCUMULATION IS ASSOCIATED WITH ACCELERATED AGING

While C1q deficiency causes autoimmunity, its elevated plasma levels signify accelerated aging. Aging is marked by a decline in tissue regeneration and repair, and in the number and dynamics of tissue stem or progenitor cells (46). At the molecular level, one observation is that progenitor cells exhibit elevated Wnt signaling in the aging tissue environment (47, 48). In aged mice, muscle stem cells exhibit increased tendency to fibroblastic differentiation (48). This was found to be conferred by a serum factor(s) in aged mice binding to the Frizzled family of cell surface receptors and causing Wnt receptor signaling (48). This Frizzled-binding protein was identified as C1q (48). Its serum level increased threefold (from 90 to 280 μg/ml) in old mice (20 months) as compared with young mice (2 months) (19).

Mechanistically, C1q binding to the Frizzled receptors causes C1s activation and activated C1s cleaves the Wnt receptor protein low-density lipoprotein receptor-related protein 6 (LRP6) to trigger canonical Wnt receptor signaling (19) (**Figure 3**). The involvement of C4 and further downstream complement

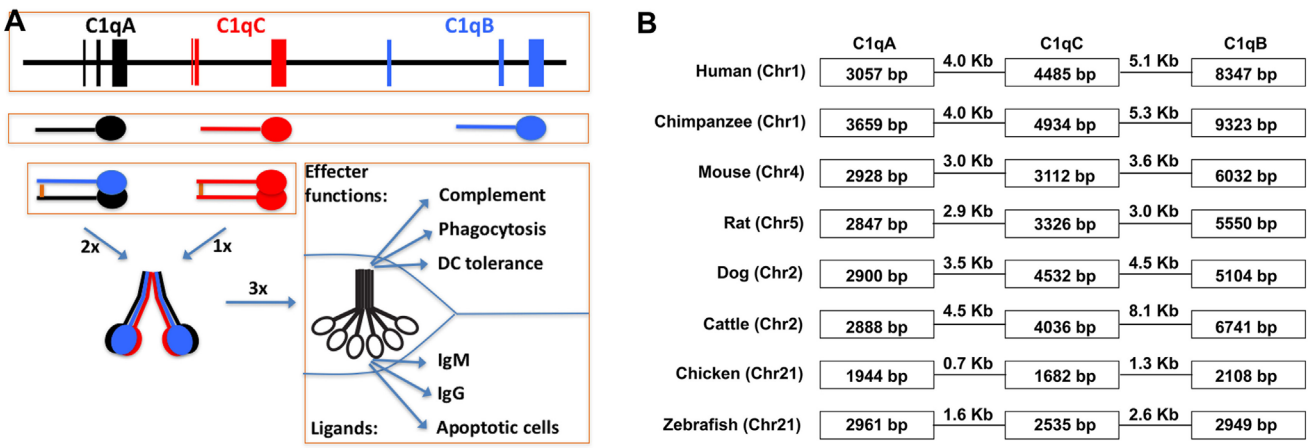

FIGURE 3 | Schematic illustration of C1q gene organization, gene transcription, and multimeric C1q assembly. (A) The three human C1q genes span approximately 25 kb on human chromosome 1. The intergenic regions are 4.0 and 5.1 kb, respectively, which are not distinguishable in size from regular introns in the C1q genes. Each of the three C1q genes contains three exons and the transcribed peptides form disulfide-linked A-B hererodimers and C-C homodimers. Each C chain in the homodimer forms a collagen triple-helix with an A-B heterodimer, and hence, two triple-helices linked by the disulfide bond in the C-C dimer. Three such ABC-CBA twin helices associate non-covalently over the N-terminal ends to form the 18-polypeptide C1q molecule. The gC1q domains are often the ligand-binding sites for C1q and the collagen triple-helices associate with the $C1r_2C1s_2$ serine protease tetramer. **(B)** Conservation of the C1q gene organization in eight different animal species. The three C1q genes in chimpanzee occupied the largest genomic space which is approx. 27 kb. In chickens, the three C1q genes occupied merely 7.7 kb with intergenic sequences of 0.7 and 1.3 kb, respectively.

components are not defined. Nonetheless, this emphasizes the rather less-studied aspect of C1-mediated cleavage of proteins outside the complement network. C1s similarly cleaves MHC class I molecule, although the C1q ligands are not defined in this context (16, 17). It appears that activation of the complement classical pathway, which involves C1s cleavage of C4 and C2, is merely one of a number of effector mechanisms downstream of the C1 complex (**Figure 3**).

Besides a distinct decline in tissue regeneration and repair, aging is also characterized by systemic elevation of the inflammatory status (49, 50). In the elderly population, plasma pro-inflammatory cytokines, IL-6 and TNF-α, and the acute phase C-reactive protein (CRP) are chronically elevated. When young (<40 years) and aged (60–81 years) populations were compared in a series of age-related parameters, including muscle mass, plasma C1q, as well as plasma IL-6, TNF-α, and CRP, the young population had clearly lower plasma C1q (80.5 μg/ml) than the aged population (161 μg/ml) (51). Interestingly, after 12 weeks of supervised resistance training intervention, plasma C1q in the elderly group decreased substantially (89.3 μg/ml) with muscle mass being significantly increased, revealing an inverse correlation between plasma C1q level and muscle mass (51). The cause for plasma C1q accumulation in the elderly group and its reduction after training is unclear in this study and a causal relationship between plasma C1q and muscle mass was also not established (51). The overall conclusion was, however, in line with C1q contribution to accelerated aging as reported in mice (19).

MECHANISM OF C1 FUNCTIONAL DIVERSITY

The mechanisms for C1 complex function in the context of complement activation and Wnt receptor signaling have been clearly documented. However, mechanistic understanding of its involvement in SLE pathogenesis remains fragmentary (**Figure 3**). Genetic deficiencies in complement proteins generally increase susceptibility to infections but mostly lack the type of strong association with SLE pathogenesis that is observed with deficiencies of C1 and its immediate substrate C4 (28). This raises the possibility that SLE pathogenesis may be related to a modular C1 activity. Depending on what C1q recognizes, C1 may have effects through the C1r/C1s proteases on various molecular/cellular processes besides the complement system. C1 activation of Wnt receptor signaling is a good example of such a modular activity (19). The degradation of apoptotic cell debris is apparently another process involving a modular C1 complex function (**Figure 3**) (9).

Since the discovery of C1q binding to apoptotic cells (9), a significant body of work has been published revolving mostly around C1q opsonization of apoptotic cells and its regulation of immune tolerance. First, C1q binding to apoptotic cells opsonizes the cell debris for effective phagocytosis (10). Second, C1q binding contributes to the immunosuppressive nature of apoptotic cells (52, 53). Third, C1q modulates dendritic cell (DC) development to induce more prominent tolerogenic features in these antigen-presenting cells (54, 55). Last, C1q inhibits IFN-α production by DCs induced by SLE autoantibodies in the form of immune complexes (56–58). IFN-α is a SLE-pathogenic cytokine, which causes autoimmunity in patients following its therapeutic administration (59, 60). IFN-α is elevated in those SLE patients who register a chronically elevated signature of IFNα-stimulated gene transcription (61–63). Inhibition of IFN-α induction by C1q potentially contributes to protection against SLE pathogenesis.

Studies that evaluate the role of C1 proteases in these processes are lacking. In fact, how C1r/C1s deficiency also causes SLE has not been investigated. There are two hypotheses that are relevant to explaining how C1 and C4 deficiencies may cause autoimmunity (64, 65). A clearance hypothesis emphasizes on the induction of autoantibodies and autoimmunity by apoptotic cellular debris, which may accumulate due to impaired clearance or excessive cell death (64). A tolerance hypothesis emphasizes on the contribution of complement to promoting self-antigen delivery to primary lymphoid organs for an effective negative selection (65). Considering that C1s cleaves intracellular antigens, it can be highly significant that the C1 complex both opsonizes apoptotic cells through C1q for effective clearance and degrades apoptotic cellular antigens through C1 proteases. Without relying on the rest of the complement system, both processes can reduce the autoantigenicity of apoptotic cell debris.

C1q was initially found to bind to apoptotic blebs, but the spectrum of C1q ligands in apoptotic cells and their contributions to C1q recognition need further delineation (9, 66). C1q appears to bind multiple regions of apoptotic cells (20). In early apoptotic cells, C1q binds to peripheral structures; however, in late apoptotic cells, it binds predominantly to the core nuclear bodies, i.e., the nucleoli (20). With purified nucleoli, C1q not only binds to these nuclear bodies but also causes C1s activation and cleavage of nucleolar proteins, e.g., nucleophosmin-1 (NPM1) and nucleolin (20). Nucleoli are highly immunogenic and contain many autoantigens (67).

This reminds an important aspect in cell apoptosis, i.e., the intrinsic proteolytic/enzymatic dismantling of intracellular structures (68). During cell apoptosis, autoantigens are cleaved and partially inactivated by endogenous proteases (69). It is possible that during late stage apoptosis, exogenous proteases and other enzymes also contribute to the antigen dismantling process. C1q recognizes multiple intracellular regions during apoptosis, including the highly immunogenic nucleoli (20). In cooperation with endogenous proteases, C1 could contribute significantly to the effective protease trimming of dead cells required to prevent their immunogenicity (**Figure 4**) (70).

C1r AND C1s SUBSTRATEOME

In the complement network, proteases are highly specific and this is essential to the directional propagation of the complement activation (5, 6). Outside the complement network, what other proteins may be cleaved by these proteases are rarely studied. With regard to C1s, it has been known for some time that it cleaves cell surface MHC class I and the secreted IGFBP5 (16–18). More recent addition to the list of non-complement

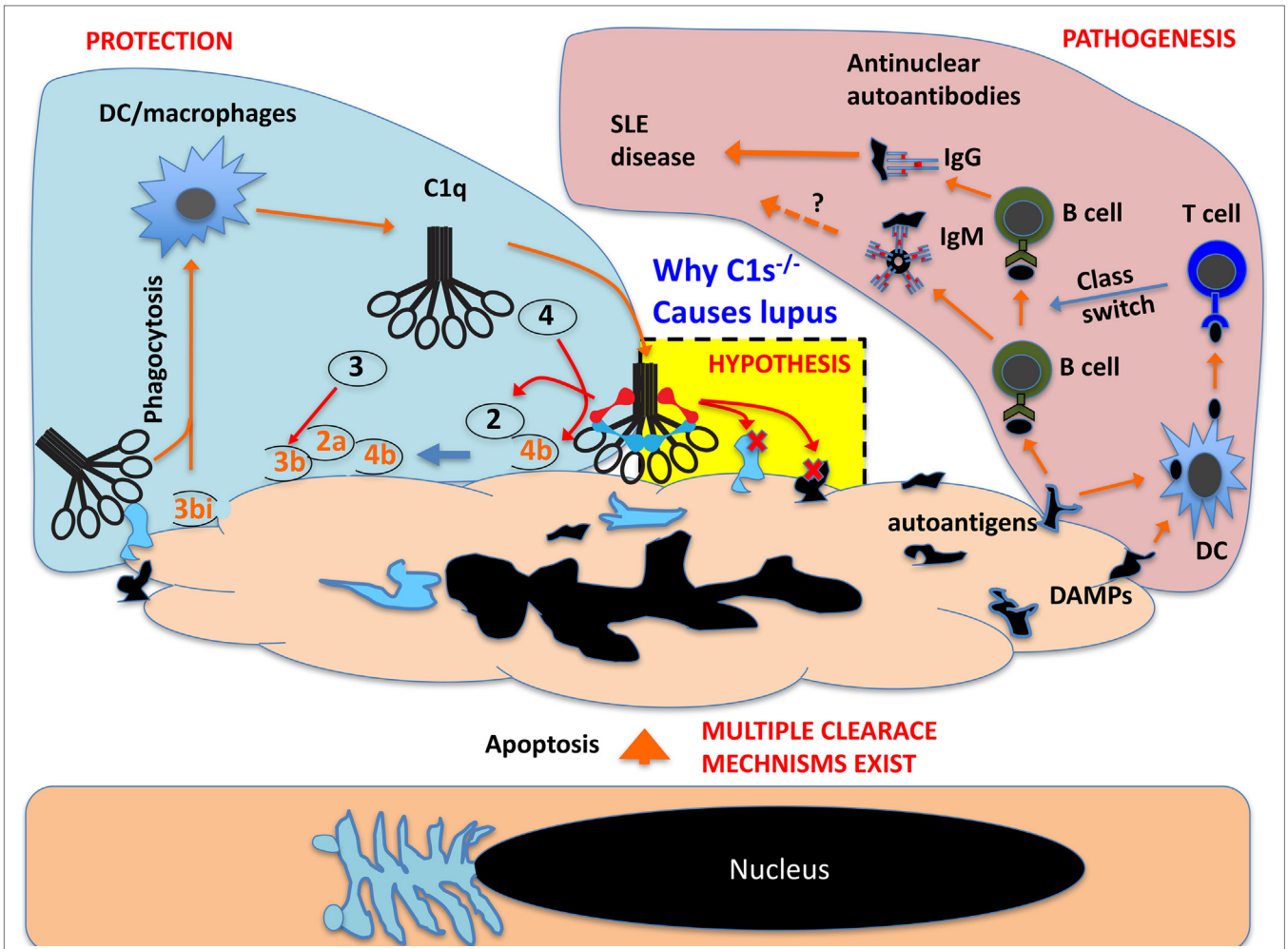

FIGURE 4 | Schematic proposal how C1 deficiency may cause systemic lupus erythematosus pathogenesis. In live cells, the nucleus and other intracellular structures are compartmentalized and excluded from complement recognition. When cells undergo apoptosis, the nucleus and other cellular structures disintegrate and, in late apoptotic cells, these fragments are recognized by C1q, which opsonize apoptotic cells for phagocytosis. This can also cause C1r/C1s activation and the activated C1s could cleave its classic substrate C4 and C2 and produce complement opsonins for phagocytosis. C1s may also cleave numerous exposed nuclear and other cellular proteins that are otherwise autoimmunogenic (autoantigens) and cause B cell production of autoantibodies. C1s may also cleave cellular proteins that are otherwise pro-inflammatory danger-associated molecular patterns (DAMPs) and activate DCs to cause B cell production of pathogenic IgG autoantibodies. C1s may not inactivate all autoantigens but effective inactivation of DAMPs can abrogate class switch of autoantibodies from IgM to pathogenic IgG.

C1s substrates includes LRP6, NPM1, and nucleolin (19, 20). In fact, the substrates of C1s can potentially be numerous based on bioinformatics predictions. Using a library of phage-displayed peptides that were designed based on the classic C1s cleavage sites on C4 and C2, Kerr et al. identified a list of C1s-cleavable peptide variants (21). Based on the conserved peptide framework, a formula was constructed that predicted numerous intracellular proteins as potential C1s substrate (21). NPM1 and nucleolin, which were found to be cleaved by C1 proteases, indeed contained multiple predicted C1s cleavage sites (20). The conjunction of a broad C1s substrateome with a diversity of C1q ligands makes the C1 complex a potentially multifaceted module that can function in a range of biological processes. C1s cleavage of intracellular proteins may be irrelevant to live cells, but this capacity could be important in the context of dead cell debris, reducing autoimmunogenicity by the inactivation of autoantigens and the destruction of danger-associated molecular patterns (DAMPs) (**Figure 4**). A recent example of this C1 protease function is the demonstrated C1s cleavage and inactivation of HMGB1, which is otherwise a nuclear DAMP (71).

C1q

The functional versatility of C1q draws support from the modularity of its structures. C1q is a large, symmetrical, and delicate posttranslational assembly resulting from complex evolutionary innovations. At one stage, the complement system was defined by merely four identifiable components, C1–C4. In 1963, C1 was first separated into three distinct subcomponents, C1q and the two proteases C1r and C1s (72, 73). For C1q, biochemical analysis revealed three types of subunit polypeptides each containing a collagenous (Gly-Xaa-Yaa)n repeating sequence over

the N-terminal half (74, 75). Similar collagen-like domains were later found in the N-terminal halves of collectins, ficolins, and some C1q/TNF-related proteins (CTRPs) such as adiponectin and saccular collagen (76–79). The collagenous regions of all these proteins form triple-helices and the C-terminal halves form globular (gC1q) domains that are clustered in three. The triple helices further conjoin at the extreme N-terminal regions to align 3–6 triple-helices in one final assembly (76, 77). In the overall "bundle of tulips" C1q assembly, the gC1q domains are peripherally extended as multivalent binding sites (74, 75). The six triple-helices in C1q form a scaffold for the tetrameric $C1r_2C1s_2$ protease complex (80). Binding of C1q to various ligands *via* the gC1q domain activates the C1r/C1s proteases and C1s triggers effector reactions through cleavage of specific substrate, which, in the complement classical pathway, are C4 and C2.

THE STRUCTURE OF C1q AFFORDS A DELICATE SCAFFOLD AND LIGAND-BINDING DIVERSITY

C1q is distinct from collectins, ficolins, and CTRPs in that it is assembled from more than one type of subunit polypeptide. The other proteins are considered largely homopolymers (76, 77). C1q is an 18-polypeptide macromolecule assembled equally from three similar but distinct subunit peptides, 6 × A, 6 × B, and 6 × C chains (74) (**Figure 2**). The C1q assembly is partially stabilized by disulfide bonds and, under denaturing conditions, the molecule crumbles into two basic structural identities, an A-B heterodimer and a C-C homodimer that are linked through N-terminal disulfide bonds (**Figure 2**). One C-C and two A-B dimers form two triple helices over the collagen-like regions (ABC-CBA) and C1q is assembled non-covalently from three such ABC-CBA structures (**Figure 2**). Therefore, despite the presence of three C1q genes, only one type of C1q is assembled. The collectins, ficolins, and CTRPs are, however, products of single genes (76, 77).

What prevented the formation of three different homopolymeric C1q molecules is not understood. The combination of divergent subunits, i.e., A, B, and C, and their extensive polymerization in C1q offers, besides a scaffold to embrace the $C1r_2$ $C1s_2$ tetramer, diversity and multiplicity of binding sites for a broad ligand repertoire. The heterotrimeric congregation of the three globular head modules (ghA, ghB, and ghC) yielding gC1q domain at the C-termini is independent of the N-terminal triple-helix (81). The three different globular head modules in the cluster exhibit differential binding preferences toward known C1q ligands (82, 83).

THE BROAD TISSUE ORIGINS OF C1q AND ITS ULTIMATE PLASMA DESTINY

A dominant source for plasma complement proteins, including C1r and C1s, are hepatocytes in the liver, but C1q is one exception for its extrahepatic origins (84). C1q was initially found produced by macrophages (85). It was later found to be produced by tissue and cultured DCs as well (86, 87). Studies on C1q gene promoters revealed active *cis*-acting elements for transcription factors PU.1 and IRF8 (26). PU.1 and IRF8, especially PU.1, is a key transcription factor that defines the macrophage and DC lineage of hematopoietic development (88). Tenner and colleagues recently clarified that, in the brain, C1q is also produced by local tissue macrophages, the microglia (89). Therefore, C1q could have evolved first as an effector molecule in macrophages or ancestral phagocytes and its association with the C1r/C1s proteases in the form of C1 complex represents a secondary evolutionary innovation.

Macrophages and DCs populate many tissues and are poorly represented in the blood circulation (90, 91). Monocytes are blood precursors of some tissue macrophages, but these cells only start to produce C1q upon differentiation into macrophages (92). How the broad and heterogeneous tissue origin of C1q and its steady plasma levels are regulated is not fully understood. Tissue macrophages, which orchestrate inflammation and antigen presentation as well as scavenge tissue debris and microorganisms, are responsive to diverse stimuli (93, 94). The complement system is concentrated in the blood and is actively recruited to sites of tissue infections or injuries. The macrophage/DC origin of C1q appears to ensure its steady state tissue distribution. Macrophages also produce C1r/C1s proteases (84). DCs also broadly populate tissues, albeit at a lower density, and also produce C1q, C1r, and C1s (86, 87, 95). This mode of C1q and C1r/C1s production stresses an important C1q or C1 function in sterile tissue homeostasis and other molecular/cellular processes.

PLASTICITY IN C1q PRODUCTION

Macrophages express a broad repertoire of scavenging and signaling receptors and exhibit a high degree of plasticity in differentiation and activation. This is reflected in the heterogeneity of tissue macrophages in their morphology and effector molecule production (91). As previously summarized, C1q production by macrophages also vary in response to microbial structures, cytokines, hormones, and drugs (66, 96). Overall, microbial structures tend to inhibit C1q production and corticosteroid hormones tend to enhance it (66). With respect to cytokines, IFN-α appears to inhibit C1q production (87), whereas IFN-γ increases C1q production by DCs/macrophages (26, 97). Local and temporal tissue fluctuation in C1q production may not prominently alter plasma C1q levels, but it can impact on local tissue homeostasis, immunity, and tolerance. This can also be of great importance in the microenvironment of tumor, where C1q seems to have a tumor-promoting function (98).

DOES C1r/C1s CLEAVE OTHER C1q-TARGETED PROTEINS?

Besides IgG and IgM, many other protein ligands have been identified for C1q (66, 99). These C1q ligands, including soluble, cell surface, normal extracellular matrix, and pathogenic amyloid proteins, often activate C1r/C1s and the complement classical pathway. It has, however, not been addressed whether the activated C1r/C1s proteases also cleave these C1q ligands or proteins

near these ligands as they cleave LRP6, IGFBP5, MHC I, NPM1, and nucleolin (16–20). In some pathophysiological contexts, C1q functions were interpreted without specific consideration to its ligands. In the postnatal central nervous system, C1q is localized to synapses and contributes to synapse elimination resembling the disposal of dead cells, which is important for the maturation of neuronal connectivity and functions (100, 101). How C1q causes the selective dismantling of synapses is unclear, but it is tempting to suggest C1q binding to selective neuronal contexts and possible involvement of C1r/C1s-mediated molecular cleavage or cell signaling as observed with the Wnt receptor (19). In excess, the same C1q-mediated synapse elimination could accelerate neurodegeneration related to aging and neuropathology (102, 103).

The scrapie pathogen, prion protein, is another C1q ligand (104, 105). C1q deficiency reduces scrapie pathogenesis (106, 107). To what extent the complement classical pathway may be involved in prion-mediated pathology is incompletely defined, but C4 is apparently activated on prion proteins and C3 depletion also reduced scrapie pathogenesis (104, 107). As C1q, C3, and C4 are all potent opsonins, a prevalent explanation is their involvement in prion transmission from the gut to the central nervous tissues. The role of activated C1r/C1s proteases in scrapie pathogenesis has not been considered.

C1q is also produced in the placenta (108, 109). At this feto–maternal interface, it was shown to mediate trophoblast invasion of the maternal decidua (108). Mechanistically, C1q was found to interact with decidual stroma, to activate trophoblast signaling, and to mediate trophoblast adhesion and migration (108). Whether C1r/C1s might play a role in this context is again unclear.

CONCLUDING REMARKS

The complement system is an intimate proteolytic cascade responding to diverse triggering factors. In infections or injuries, the full impact of its activation is realized by three closely related effector reactions: inflammation, opsonization, and lysis (5, 6). The C3a and C5a anaphylatoxins recruit and activate phagocytes and other inflammatory leukocytes at sites of tissue infections or injuries. The membrane attack complexes cause cellular lysis. The C4b, C3b, and the further proteolytic fragments opsonize complement-reacted targets for effective phagocytic clearance (**Figure 1**). However, this article highlights that C1 complex may function as a module, independent of the rest of the complement network, to participate in other molecular/cellular processes.

Serine proteases are core components of the complement infrastructure and their sequential activation is at the heart of the formation of hierarchical proteolytic or lytic protein complexes. In the context of the complement network, these are highly specific proteases, e.g., C1r only cleaves C1s and C1s only cleaves C4, C2, and C1 inhibitor. The finding that the C1 proteases also cleave a growing list of non-complement proteins, including LRP6, MHC I, IGFBP5, NPM1, and nucleolin, supports a multifaceted, modular function for C1 complex. In this functional C1 module, C1q recognizes targets in various molecular/cellular processes and the C1r/C1s proteases bring about the effects by cleaving substrate in these molecular/cellular processes. Modular functions may also be found in other complement proteases such as factor B and MASPs.

AUTHOR CONTRIBUTIONS

JL initiated the article and contributed to the framework and major details of the final version. UK provided critical and substantial inputs to the overall scope and detailed content. This is a joint project by the two authors.

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Is the A-Chain the Engine That Drives the Diversity of C1q Functions? Revisiting Its Unique Structure

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The immunopathological functions associated with human C1q are still growing in terms of novelty, diversity, and pathologic relevance. It is, therefore, not surprising that C1q is being recognized as an important molecular bridge between innate and adaptive immunity. The secret of this functional diversity, in turn, resides in the elegant but complex structure of the C1q molecule, which is assembled from three distinct gene products: A, B, and C, each of which has evolved from a separate and unique ancestral gene template. The C1q molecule is made up of 6A, 6B, and 6C polypeptide chains, which are held together through strong covalent and non-covalent bonds to form the 18-chain, bouquet-of-flower-like protein that we know today. The assembled C1q protein displays at least two distinct structural and functional regions: the collagen-like region (cC1q) and the globular head region (gC1q), each being capable of driving a diverse range of ligand- or receptor-mediated biological functions. What is most intriguing, however, is the observation that most of the functions appear to be predominantly driven by the A-chain of the molecule, which begs the question: what are the evolutionary modifications or rearrangements that singularly shaped the primordial A-chain gene to become a pluripotent and versatile component of the intact C1q molecule? Here, we revisit and discuss some of the known unique structural and functional features of the A-chain, which may have contributed to its versatility.

Keywords: Complement, classical pathway, C1q, A chain, charge pattern recognition, C1q receptor

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THE COMPLEX STRUCTURE OF C1q

C1q is the first subcomponent of the complement classical pathway. In addition to its complement activation mediated immune functions, it has a broad range of developmental homeostatic functions that are not dependent on its ability to activate the classical pathway [reviewed in Ref. (1)]. The functional versatility of C1q depends on several unique structural and functional properties (1–3). It is made up of three chains, A, B, and C, which are the product of three distinct genes, found highly clustered and aligned 5′⇒3′, in the same orientation, in the order A–C–B on a 24 kb stretch of DNA on chromosome 1p at position 36.12 (4, 5). Each chain contains an N-terminal collagen-like region and a C-terminal globular head region. There are 18 chains in the intact C1q molecule: 6A, 6B,

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

and 6C, which are arranged first as a single heterotrimeric strand comprising of A, B, and C, in which the A chain and B chain within the strand are covalently linked to each other, whereas the C-chain of one strand which is non-covalently associated with the AB dimer, nonetheless forms a covalent link with the C chain of a neighboring ABC strand to form an ABC-CBA doublet. Three such doublets are then held together with non-covalent bonds to give rise to the well-recognized hexameric structure of C1q. The globular "heads" of each ABC strand are linked *via* six collagen-like "stalks" to a fibril-like central region resulting in two unique structural and functional domains: the collagen-like region (cC1q) and the globular "heads" or domains (gC1q) (6, 7). Each of the gC1q domains is a heterotrimeric structure made up of each of the individual chains (ghA, ghB, and ghC). What has become apparently clear is the fact that each of the gh domains is capable of recognizing a gh-specific ligand independent of the other gh domains (3, 8, 9). Therefore, assuming that each of the gh domains recognizes a single target or ligand, the C1q molecule can recognize and bind simultaneously six individual molecular patterns, making it one of the most efficient, and versatile pattern recognition molecules.

The crystal structure of the heterotrimeric gC1q domain revealed a compact jellyroll β-sandwich fold similar to that of the multifunctional tumor necrosis factor (TNF) family of proteins (10, 11). This suggested that C1q not only diverged from a primordial ancestral gene template of the innate immune system that gave birth to the TNF-α and other C1q-like proteins, but also retained some of its ancestral "cytokine-like" functions (2, 10). Therefore, C1q could be considered as a prototype "complekine," i.e., complement protein with cytokine-like activity, which is capable of mimicking some, if not all, functions of the TNF family of proteins, including the induction of cytokines (IL-6 and IL-8) and chemokines (e.g., MCP-1) that orchestrate a myriad of a rapidly expanding list of pathophysiological processes (12, 13).

There is also an abundance of clinical evidence, which shows that genetic deficiency in C1q is associated with a wide range of clinical syndromes closely related to SLE, with rashes, glomerulonephritis, and CNS disease as well as other autoimmune diseases (14). In addition, C1q also can have a major role in tumor growth and progression (15–19). The role of C1q, being a part of tumor microenvironment, has appeared to be complex so far. In some reports, it has been shown to be protumorigenic (15–17), whereas there are recent reports of antitumor activities of C1q in the case of prostrate (18) and ovarian cancers (19).

Although individuals with congenital C1q deficiency constitute only a small cohort of patients, this strong association nonetheless implicates an important role for complement in general, and C1q in particular, in the development of SLE and other autoimmune diseases (20–24). What is perplexing, however, is the fact that among the C1q deficiencies, the A-chain of C1q should take center stage in significance as homozygous deficiency or mutation in the A-chain is almost invariably associated with various diseases (20–24). The mutation in the A-chain is due to a homogeneous mutation in which the C to T transition in codon 186 of exon 2 results in Gln-to Stop (Q186X) substitution. The question is: what are the structural signatures that make the C1q-A chain such a powerful susceptibility biomarker of these diseases? It is worth noting that although the most prevalent mutation is the C1qA, Gln208X mutation, there are other mutations in B and C chains too (25).

STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF THE C1q A-CHAIN

The genes encoding the three chains of C1q are highly conserved from zebrafish to human. Phylogenetic analysis also intimates that the C1qA, C1qB, and C1qC may have originally been generated by gene duplications from a single copy of an ancestral C1qB gene, since the latter is found in the same branch as amphioxus C1q, which is an earlier lower vertebrate than zebrafish (26). Furthermore, the IgG binding properties between fish and mammalian C1q show no difference since substitution of human C1q by fish C1q has the same activity, suggesting that the IgG or IgM recognizing properties have remained conserved throughout the evolutionary history (26, 27). However, more recent studies have shown that there is a preferential binding of the gC1q modules when it comes to IgG binding. Whereas the gC1qA (or ghA) module binds aggregated IgG and IgM in a similar manner, gC1qB (ghB) binds aggregated IgG in preference to IgM (28). The functional preferences of the gC1q domains, therefore, may reflect an evolutionary structural adaptation that resulted in recent history.

In an elegant and in depth review, Trinder et al. (29) analyzed the structural and functional correlates that distinguish the A-chain from the B- and C-chains. *First*, while the B and C chains are highly conserved, the A-chain is not. This fact alone should support the notion that the A-chain developed to be functionally adaptable throughout evolution. *Second*, various types of cells including macrophages and dendritic cells among a long list of others, synthesize the C1q molecule. The cell-associated molecule in turn, is anchored in the membrane *via* a 22 amino acid long leader peptide, which is found only in the A-chain (29). *Third*, the A-chain contains several antigen recognition sites (**Figure 1**), but in particular, possesses one major (aa 14–26) and one minor (aa 76–92) promiscuous region (29), which serve as a binding site for a wide range of non-immunoglobulin antigens including lipopolysaccharide, C-reactive protein (CRP), DNA, heparin, fibronectin, monosodium, urate crystals, amyloid P component, von Willebrand factor (30) as well as bacterial and mitochondrial membranes (29–42). Importantly, this A chain region has also been shown to bind specifically by SLE patients' sera compared to serum derived from healthy control (43). Although recent studies have suggested that the interaction site for CRP is located in the gC1q rather than the cC1q (44–46), it is plausible to assume that certain molecules could actually bind to multiple regions of the A-chain. Regardless, these non-immunoglobulin antigens have been shown to activate the classical pathway by binding to the cC1q region of the A-chain rather than to the globular heads (29–42). In addition, the A-chain contains a collagen-type II-like sequence comprising of residues 26–34, which has been shown to suppress collagen type-II-induced arthritis in a mouse model (47). Interestingly, this same region is also predicted to be a potential MHC class II binding site (48). However, little is known about the significance of this finding but may have potential implications

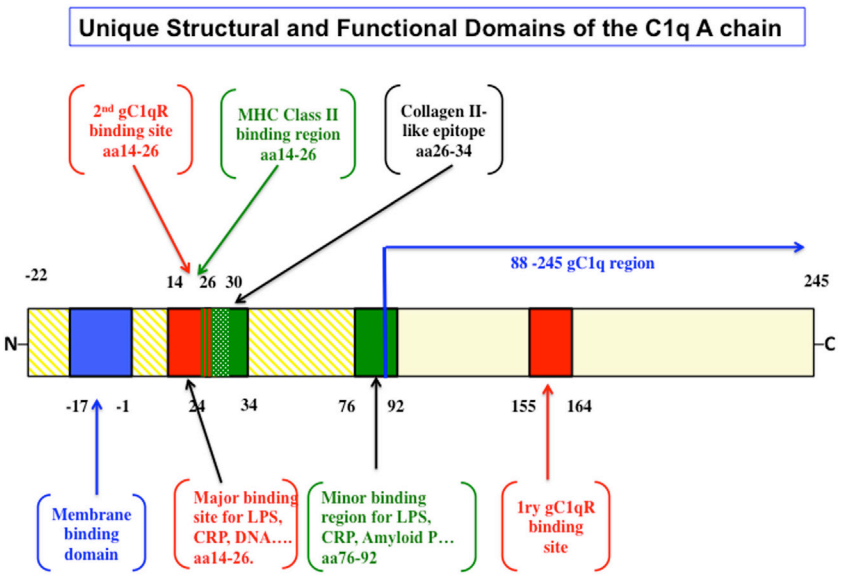

FIGURE 1 | The structural and functional correlates of the C1q A-chain. The intact C1q molecule is anchored to the cell membrane by a leader peptide in the A chain. The major and minor ligand bind sites as well as the putative MHC class II binding domain are highlighted. Although the major gC1qR-binding domain spans residues 155–164, unexpected sites in the promiscuous collagen domain spanning residues 14–26 and another at 76–92 have also been shown very recently [the figure is adapted from Ref. (29)].

in autoimmunity and tolerance (48), especially since C1q has been shown to keep monocytes in a predendritic or immature phenotype, thus ensuring that unwarranted DC-driven immune response does not occur, a fact that is relevant to the development of SLE (49). Finally, although it is found only in the mouse, and not in the human C1q A-chain, the presence of an RGD sequence may also explain why human C1q still retains its ability to support adhesion of normal endothelial cells and fibroblasts (50–53) in a manner that is inhibited by an RGD peptide but not an RGE (51). Very recently, Agostinis et al. have shown that C1q can act as a bridge between hyaluronic acid (HA), an abundant matrix component of the tumor microenvironment, and the HA receptor on tumor cells, i.e., CD44, thus inducing considerable proliferation of primary tumor cells derived from malignant pleural mesothelioma (MPM) (17). Curiously, the A-chain of the globular region of C1q bound specifically and differentially to a range of LPS-free HA, leaving C-chain to liaise with MPM cells.

Thus, although it may be overly simplistic to suggest that the A-chain is the functional anchor of the C1q molecule, it appears to be clear that of the three chains, the A-chain has singularly undergone systematic and adaptable molecular evolution. Whether the selection of the A-chain to evolve as a master orchestrator of C1q functions was by design or serendipity, or whether the B- and C-chains are also undergoing similar evolution albeit at a much slower rate, are questions still for the future.

AUTHOR CONTRIBUTIONS

BG and EK wrote the first draft; UK and EP revised and edited the manuscript.

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ORIGINAL RESEARCH
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Fc-Galactosylation of Human Immunoglobulin Gamma Isotypes Improves C1q Binding and Enhances Complement-Dependent Cytotoxicity

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Binding of the complement component C1q to the CH2 domain of antigen-bound immunoglobulin gamma (IgG) activates the classical complement pathway and depends on its close proximity to Fc fragments of neighboring antibodies. IgG subclasses contain a highly conserved asparagine 297 (N)-linked biantennary glycan within their CH2 domains, the core structure of which can be extended with terminal galactose and sialic acid residues. To investigate whether Fc-glycosylation regulates effector functions of human IgG subclasses, we cloned the antigen-binding region of the CD20-specific monoclonal antibody rituximab into IgG isotype expression vectors. We found that Fc-galactosylation enhances the efficacy of CD20-targeting complement-fixing antibodies for C1q binding and complement-mediated tumor cell lysis. Increased efficacies were restricted to IgG1 and IgG3 subclasses indicating that Fc-galactosylation alone is not sufficient for IgG2 and IgG4 to acquire complement-fixing properties. Addition of terminal galactose to the N-glycan specifically improved binding of C1q without changing antigen- and FcγRIIIa-binding affinities of IgG isotypes. These data indicate that Fc galactosylation can be harnessed to enhance the complement-activating properties of IgG1 and IgG3 antibodies.

Keywords: c1q, complement system proteins, antibodies, monoclonal, glycan, IgG subclasses

INTRODUCTION

Immunoglobulin gamma (IgG) antibodies are preeminent effector proteins of the immune system. The bimodal architecture of IgG allows for simultaneous recognition of antigen (by the antigen-binding fragment, Fab) and the initiation of IgG effector functions such as recruitment and activation of leukocytes and antibody-dependent cell-mediated cytotoxicity (ADCC) through interaction with FcγRs as well as complement activation through binding of the constant, crystallizable fragment (Fc) binding of C1q. Human IgG is subdivided in the four isotypes IgG1 to IgG4, numbered based on their abundance in serum (1). Despite being more than 90% identical in amino acid sequence, IgG isotypes differ in key functional regions responsible for flexibility, FcγR binding, and complement fixation (2).

All IgG subclasses contain a highly conserved asparagine-linked (N-)oligosaccharide located in the CH2 domain of the Fc region. Presence of this glycan serves important functions in protein

folding and post-translational quality control mechanisms and is essential for antibody-mediated effector functions (3, 4). The biantennary core glycan structure, which is composed of two N-acetylglucosamines (GlcNAc) and three mannose residues, can be further decorated with fucose, bisecting GlcNAc and terminal GlcNAc, galactose, and sialic acid. The highest degree of variability among IgG antibodies stems from the presence or absence of galactose with around 40% containing one galactose, 20–40% two galactoses, and the remainder none (5–7).

Presence or absence of distinct N-glycan residues such as fucose and sialic acid can dramatically alter pro- and anti-inflammatory IgG activities. Removal of the fucose residue from human IgG1 increases antibody-dependent cell-mediated cytotoxicity (ADCC) through improved affinity for Fcγ receptor IIIa (FcγRIIIa) (8, 9). We have previously shown that IgG Fc sialylation of human monoclonal IgG1 molecules impairs their efficacy to induce complement-dependent cytotoxicity (CDC) (10). Here, we determined whether Fc galactosylation of human IgG isotypes (IgG1–4) regulates antibody effector functions.

RESULTS

Generation of Degalactosylated and Galactosylated IgG Isotypes Targeting CD20

To test the impact of Fc galactosylation of human IgG isotypes on target cell depletion, we first cloned the sequence encoding the heavy chain (HC) and light chain (LC) antigen-binding regions of the monoclonal CD20-targeting antibody rituximab into human IgG1–4 HC and kappa LC expression vectors. Plasmids were cotransfected in HKB11 cells and antibodies were purified from the supernatant using protein G columns. Next, we either treated the antibodies with recombinant galactosidase to completely remove galactose or with β-1,4-galactosyltransferase in the presence of uridine diphosphate (UDP-) galactose to obtain galactosylated antibodies. After repurification, antibodies were analyzed for purity and integrity by gel electrophoresis and immunoblotting with the galactose-specific *Erythrina Cristagalli* lectin to confirm the successful generation of glycovariants (**Figure 1**).

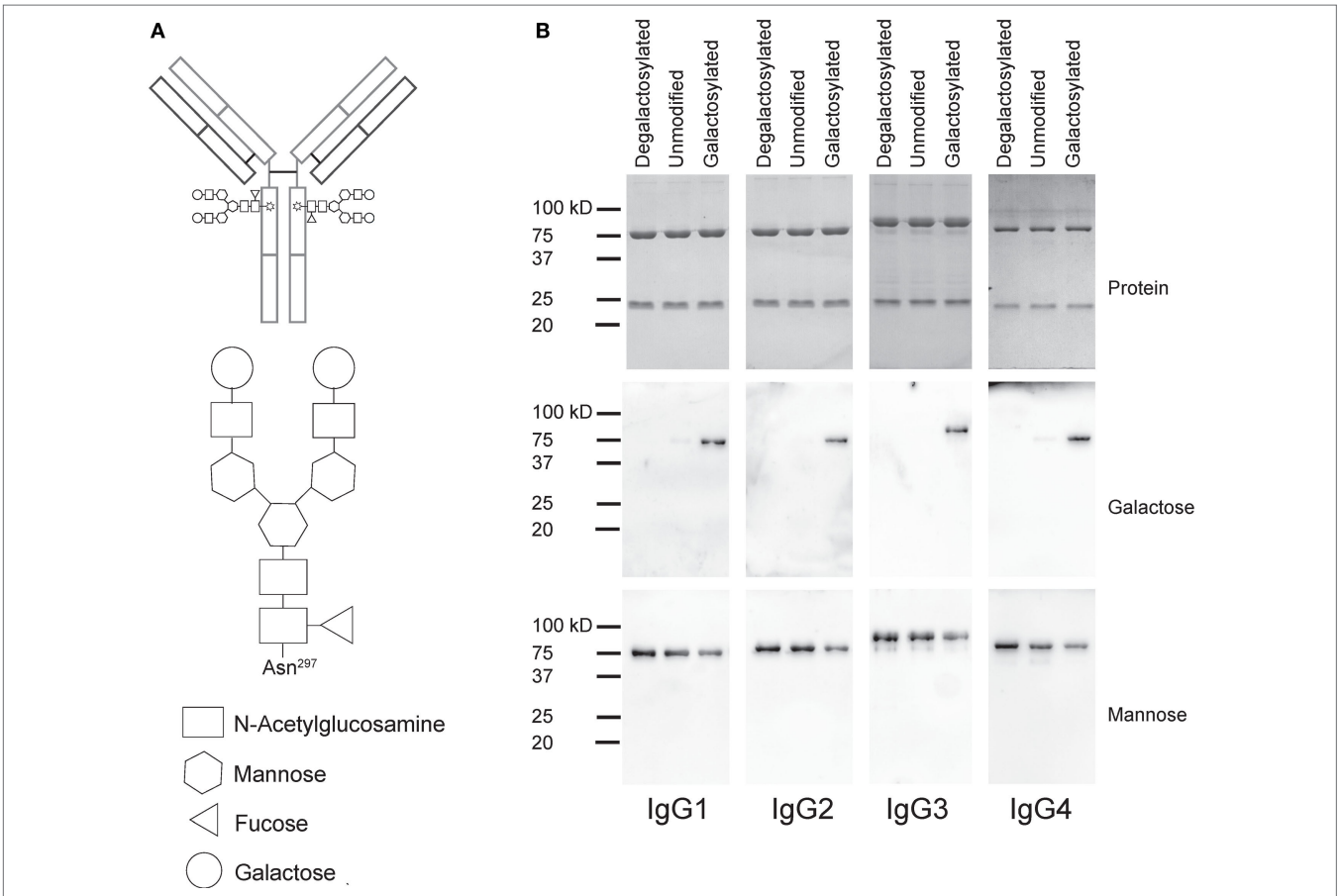

FIGURE 1 | Generation of degalactosylated and galactosylated immunoglobulin gamma (IgG) isotypes derived from rituximab. **(A)** Schematic depiction of an IgG antibody with its two Fc N-glycans (up) and detailed glycan composition of a fully galactosylated IgG-Fc N-glycan. **(B)** Gel electrophoresis and coomassie staining to detect total protein showing heavy and light chains of antibodies (up) and immunoblots using galactose- (*Erythrina Cristagalli* lectin) or mannose- (*Lens Culinaris* agglutinin) specific lectins.

Addition or Removal of IgG-Fc Galactose Does Not Affect Antigen Binding

Modifications in the Fc domain may change the structural properties of the antibody, potentially leading to changes in its antigen-binding region. To determine antigen-binding affinities of IgG isotype glycovariants, we titrated the antibodies on CD20 expressing human Raji-Burkitt's lymphoma cells and analyzed binding by flow cytometry (**Figure 2**). For each isotype, galactosylated and degalactosylated glycovariants did not differ in their antigen-binding characteristics.

IgG-Fc Galactosylation of IgG1 and IgG3 Isotypes Increases C1q Binding and Complement-Dependent Cytotoxicity

The efficacy of IgG isotype-derived glycovariants to induce complement-dependent cytotoxicity (CDC) was determined in Burkitt's lymphoma-derived Raji cells in the presence of active complement (human serum). Rituximab-derived IgG3 glycovariants showed the highest efficacy for CDC, followed by IgG1 (**Figure 3**). Glycovariants of IgG2 and IgG4 isotypes did not induce CDC. Galactosylation increased CDC mediated by both IgG1 (26% reduction of EC50) and IgG3 (13% reduction of EC50) but did not provide IgG2 and IgG4 with *de novo* ability to lyse target cells (**Figure 3**). To investigate the mechanism by which Fc-galactosylation impacts CDC, we determined the C1q binding affinities and kinetics of galactosylated and degalactosylated antibody variants (10). Incubation of CD20-expressing Raji cells in the presence of human serum depleted for C5, an essential component of the complement cascade, which allows to analyze the binding of members of the complement cascade to target cells while preventing cell lysis (10), led to rapid binding of C1q (**Figure 4**). Fc-galactosylation substantially enhanced the antibodies' capacity to bind C1q for IgG1 and IgG3 isotypes (**Figure 4**). These data indicate that the addition of terminal galactose to the Fc-glycan enhances cell-depleting efficacies of human IgG1 and IgG3 isotypes through increased C1q binding.

Fc-Galactosylation Does Not Increase IgG:FcγRIIIa-Binding Affinities

Rituximab depletes B cells through a combination of CDC and antibody-dependent cell-mediated cytotoxicity (ADCC), which requires antibody binding to the human-activating FcγRIIIa (CD16) (10, 11). Absence of the IgG-Fc core fucose increases binding to FcγRIIIa (8, 12), a finding increasingly being used to improve the efficacy of therapeutic antibodies (13–15). Two studies reported that Fc-galactosylation results in a slight, albeit not statistically significant increase in FcγRIIIa-binding affinity and ADCC activity (16, 17). A more recent study confirmed that afucosylated glycoforms show higher binding affinities for FcγRIIIa, while Fc-galactosylation did not significantly impact FcγRIIIa binding (18). To investigate whether Fc-galactosylation of IgG isotype glycovariants increases binding affinities of IgG isotypes for FcγRIIIa, thereby potentially enhancing the antibodies' ability to induce ADCC, we titrated IgG glycovariants onto CHO-derived cell lines recombinantly expressing FcγRIIIa. FcγRIIIa binding was strongest for IgG1 and IgG3 isotypes

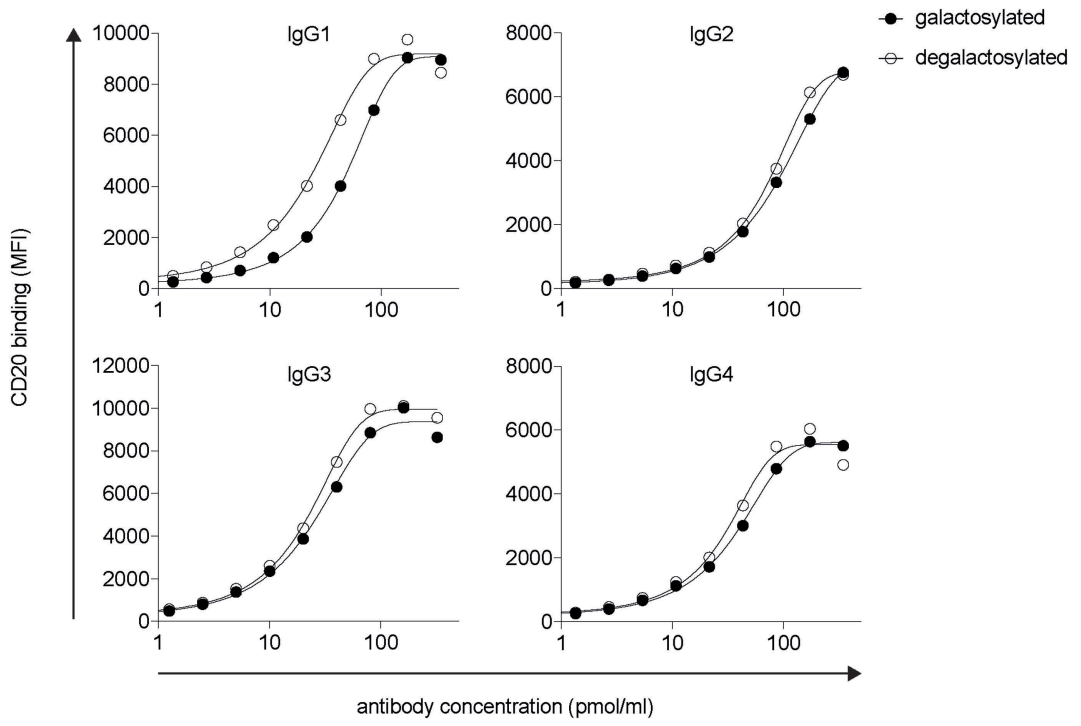

FIGURE 2 | Fc-galactosylation does not increase CD20 binding of rituximab-derived IgG isotypes. Target antigen recognition by galactosylated and degalactosylated human IgG1–4 isotypes specific for CD20 analyzed by flow cytometry *via* titration on CD20+ Raji cells. MFI, median fluorescence intensity; IgG, immunoglobulin gamma.

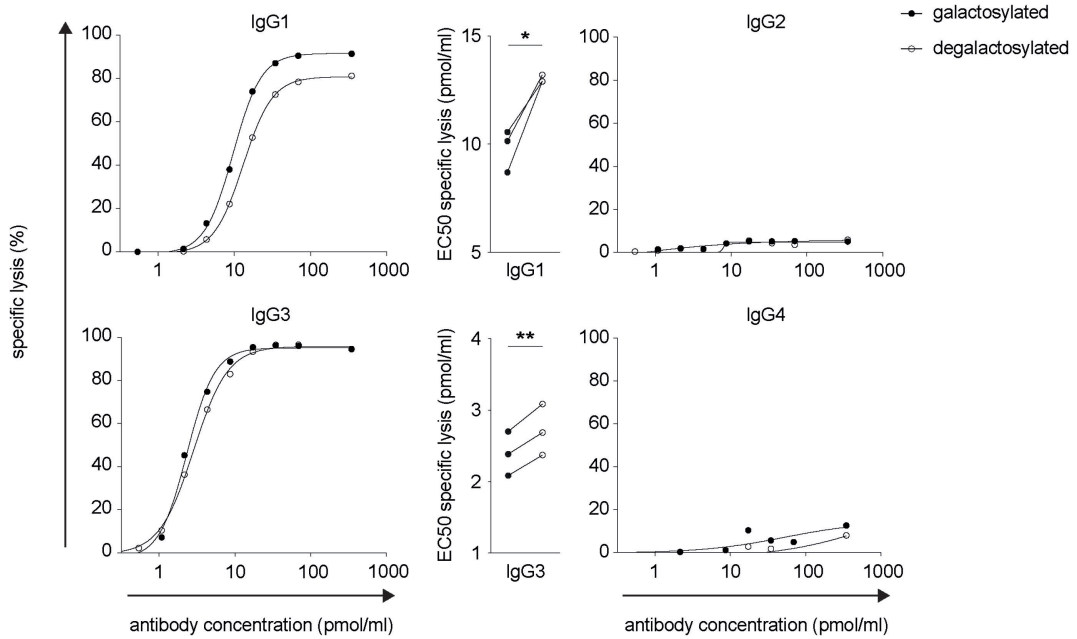

FIGURE 3 | Increased CDC of rituximab-derived immunoglobulin gamma 1 (IgG1) and IgG3 but not IgG2 and IgG4 upon Fc-galactosylation. Complement-dependent lysis of CD20+ target cells in the presence of galactosylated or degalactosylated human anti-CD20 IgG isotypes. Exemplary lysis curves and EC50 values of three independent experiments are shown. Statistical analysis: paired two-tailed Student's *t*-test $*p < 0.05$, $**p < 0.01$.

(**Figure 5**). IgG-Fc galactosylation showed, however, no effect on FcγRIIIa-binding affinities for any of the IgG isotypes tested (**Figure 5**). Thus, while increasing the antibodies' affinity for C1q binding and their efficacy to induce CDC, Fc-galactosylation did not change the affinity of human IgG isotypes for FcγRIIIa which, upon ligation, mediates ADCC.

DISCUSSION

Initiation of the classical complement cascade trough binding of C1q is a potent proinflammatory mechanism by which IgG antibodies trigger immune responses during infection and its deregulation causes tissue damage in a wide array of human inflammatory, degenerative, and autoimmune diseases (19). Our study shows that Fc-galactosylation enhances the efficacy of complement-fixing IgG isotypes to induce CDC through improved binding of C1q.

The antibody-dependent classical complement pathway is initiated if the C1 complex, formed by the multimeric pattern recognition molecule C1q and the modular proteases C1r and C1s, docks on antigen-bound IgG (20). C1q binds to monomeric IgG with very low affinity but antigen-driven antibody clustering allows for the formation of IgG hexamers that bind C1q with high avidity and promote efficient complement activation (21). The N-glycan resides in the CH2 domain, which is required for C1q binding (22). Sites on the surface of human IgG1 that constitute the C1q-binding epicenter are conserved in human IgG isotypes that are deficient in C1q binding and it has therefore been suggested that the composition of the N-glycan might be critical for the antibodies' conformation and its ability to bind C1q (23). Each biantennary oligosaccharide chain extends one arm toward the CH2–CH3 interface region and the other arm into the space between the CH2 domains, resulting in multiple interactions with the surface of the CH2 domain and the glycan of the opposing CH2 domain, respectively (4). Indeed, optimal C1 activation requires the presence of the Fc-glycan since C1q-mediated effector functions are compromised or lost in aglycosylated or deglycosylated IgGs (24–26). The effect of deglycosylation on reducing C1q binding has recently been attributed to its inhibition of IgG hexamerization *via* modulation of IgG Fc:Fc interactions rather than reduction of direct C1q-Fc-binding affinities (27). Based on these data and our results, we suggest that Fc-galactosylation modulates Fc:Fc interactions for antigen-bound IgG, thereby improving binding of C1q and increasing the antibodies' ability to induce classical complement activation and CDC.

We systematically investigated whether Fc-galactosylation facilitates C1q binding and CDC effector functions across all human IgG isotypes. For murine IgG2b and IgG1, it has been demonstrated that addition of terminal galactose increases binding of C1q (28). While presence of terminal galactose enhanced complement activation by CD20 targeting, C1q-fixing human IgG1 and IgG3 isotypes, IgG2 and IgG4 remained deficient in initiating the classical complement cascade indicating that Fc-galactosylation alone is not sufficient for IgG2 and IgG4 to acquire complement-fixing properties.

Rituximab and therapeutic monoclonal antibodies (mAbs) that target tumor cells *via* ADCC or CDC are approved for the treatment of various cancers (29). B-cell depletion by CD20-targeting antibodies is also widely used for the treatment of autoimmune diseases (30). However, some patients do not sufficiently

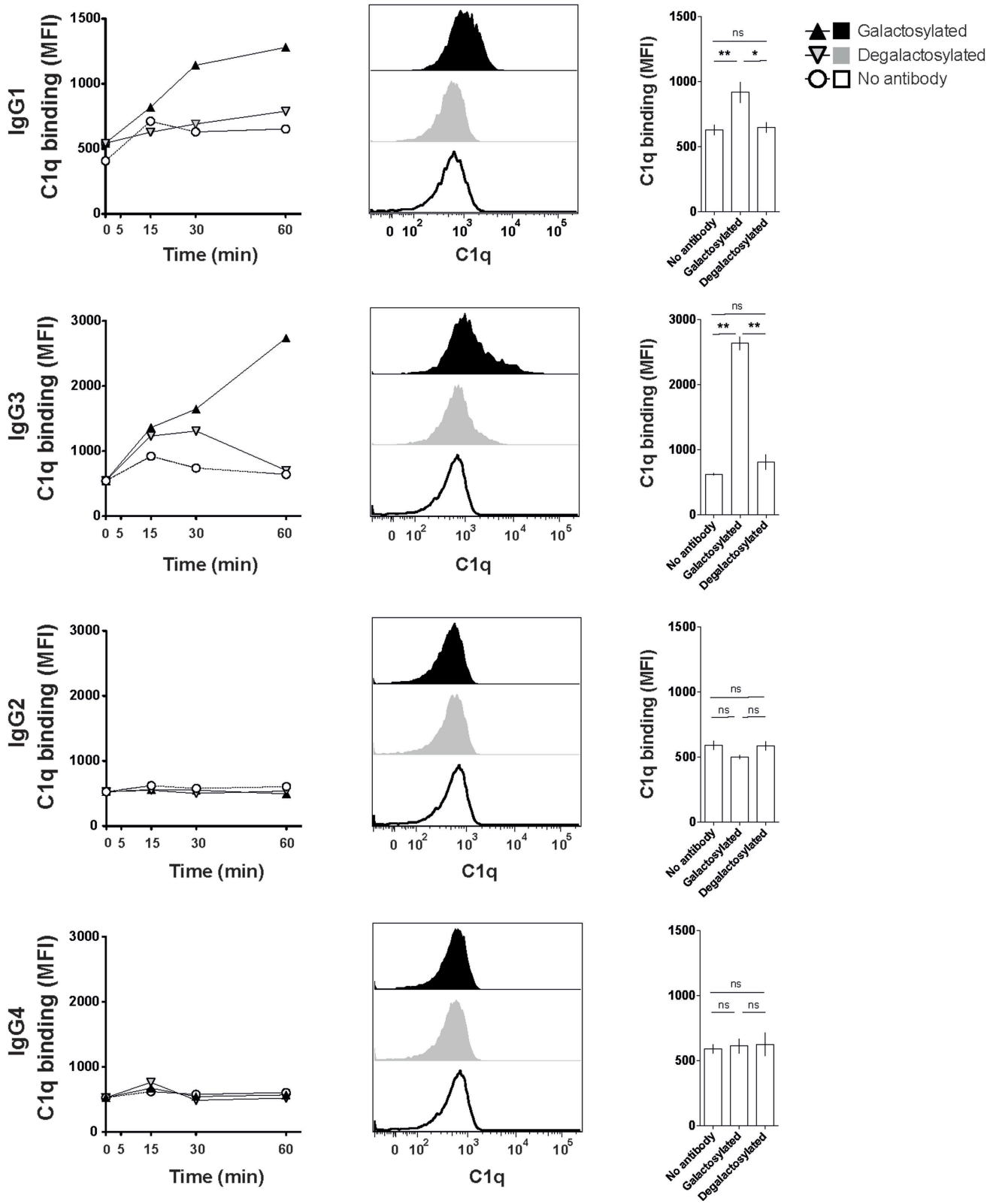

FIGURE 4 | Increased C1q binding of Fc-galactosylated rituximab-derived immunoglobulin gamma 1 (IgG1) and IgG3. Kinetic of C1q binding to CD20+ Raji cells in the presence of galactosylated or degalactosylated glycovariants of human IgG1–4. Exemplary C1q-binding curves (left), flow cytometry histograms of C1q binding after 60 min (center) and statistics at time point 60 min of at least three independent experiments. Statistical analysis: unpaired two-tailed Student's *t*-test, mean ± SEM, ns, not significant, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. MFI, median fluorescence intensity.

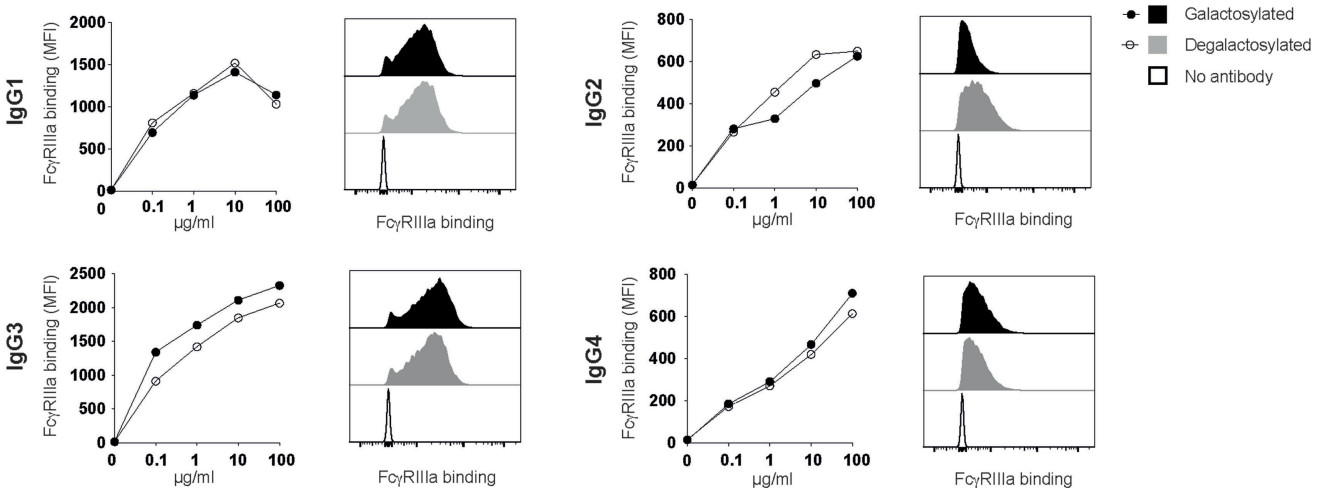

FIGURE 5 | Immunoglobulin gamma (IgG) binding to FcγRIIIa does not require Fc-galactosylation. Binding of galactosylated and degalactosylated IgG isotypes to CHO cells transfected with FcγRIIIa was analyzed by flow cytometry. Binding curves and flow cytometry histograms at 10 µg/ml are shown.

respond to rituximab therapy (31, 32) and improved versions of B cell depleting antibodies have been developed to increase ADCC activity and improve clinical efficacy (33, 34). Our data indicate that Fc-galactosylation, in addition to the established effect of defucosylation on ADCC, increases target cell cytotoxicity through enhancing CDC. Fc-galactosylation specifically improved binding of C1q to human IgG1 and IgG3 without changing antigen-binding affinities. Our data therefore indicate that Fc-galactosylation should be harnessed in glycoengineering therapeutic antibodies for effective antibody-dependent complement activation and target cell killing.

MATERIALS AND METHODS

Buffers and Reagents

Human serum complement (Cat. No.: A100; Lot No.: 035997) and C5-depleted human serum (Cat. No.: A501; Lot No.: 031795) were purchased from Quidel® Corporation. Accutase was purchased from StemCell Technologies Inc. (Cat. No.: 07920), Gibco™ RPMI-1640, HEPES (Cat. No.: 52400025), penicillin–streptomycin (P/S; 5,000 U/ml; Cat. No.: 15070063), LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Cat. No.: L34957), LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Cat. No.: L10119), and TO-PRO-3 Stain (Cat. No.: T3605) were purchased from Invitrogen. Fetal calf serum (FCS) was purchased from Sigma-Aldrich (Cat. No.: F7524-500M; Lot No.: 051M3395) and from Bioswisstech (Cat. No.: S0615; Lot No.: 1047D). β1-4 galactosidase (Cat. No.: 345806-50MIU) and UDP-galactose (Cat. No.: 670111-50MG) were purchased from Merck Millipore Corporation, Calbiochem®. Protein-G sepharose was purchased from GE healthcare (Cat. No.: 17-0618-01). Biotinylated *Erythrina Cristagalli* lectin (Cat. No.: B-1145) and *Lens Culinaris* Agglutinin (Cat. No.: B-1045) were purchased from Reactolab. Phosphate-buffered saline (PBS; 2.68 mM KCl, 8 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, pH adjusted to 7.3) was produced in-house.

Antibodies and Streptavidin

Monoclonal mouse anti-human C1q (Cat. No.: A201) and biotinylated anti-human C1q (Cat. No.: A700) were purchased from Quidel® Corporation. Polyclonal rabbit anti-mouse IgG (H + L)-Alexa Fluor 488 (Cat. No.: A-11059) was purchased from Invitrogen. PE-conjugated streptavidin (Cat. No.: 405203) was purchased from Biolegend.

CDC Assay

Raji cells were used as $CD20^+$ target cells to assess CDC. Accordingly, 7×10^4 Raji cells were cultivated in RPMI-1640 containing P/S (50 U/ml) in a humidified incubator (37°C, 5% CO_2) in 96-well V-bottom plates. Cells were incubated with titrated concentrations of the respective galactosylated and degalactosylated anti-CD20 antibody isotypes. After 30 min, human serum complement was added to a final concentration of 5% and incubation was continued for another 12 h. Thereafter, cells were washed twice by adding 200 µl cold PBS and centrifugation at 400 × *g* and 4°C for 5 min. Cells were resuspended in cold PBS and TO-PRO3 stain (final concentration of 200 nM) was added to detect dead cells. All samples were analyzed on a BD FACSCanto-II using FACSDiva v6.1.3 software and FlowJo software v9.3.1 (Tree Star Inc.). Specific lysis was calculated as percent increase in dead cells compared to spontaneous lysis in the absence of an anti-CD20 antibody. EC50 values were calculated *via* a non-linear regression in GraphPad Prism 5.

C1q-Binding Assay

A total of 2×10^5 Raji cells were incubated with 10 µg/ml with galactosylated and degalactosylated anti-CD20 antibody isotypes in RPMI-1640 containing 1% P/S (50 U/ml) and 1% C5-depleted human serum for 5, 15, 30, or 60 min in a humidified incubator (37°C, 5% CO_2). After incubation with the different anti-CD20 antibody isotypes, cells were washed twice with 200 µl cold PBS, and C1q binding was determined by adding biotinylated anti-C1q antibody (25 µg/ml in PBS) for 1 h on ice followed by washing

twice with 200 µl cold PBS and incubation with PE-conjugated streptavidin (1:400 in PBS) and dead cell stain (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit; 1:250 in PBS) for 20 min. After washing twice with 200 µl PBS, samples were analyzed on a BD FACSCanto-II using FACSDiva v6.1.3 software and FlowJo software v9.3.1 (Tree Star Inc.).

CD20-Target-Binding Assay

Raji cells were maintained in RPMI-1640 containing P/S (50 U/ml) and 10% FCS in a humidified incubator (37°C, 5% CO_2). 1.2×10^5 Raji cells and anti-CD20 antibody isotypes and glycovariants were incubated for 25 min on ice. Dead cells were excluded using LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit. Cells were washed twice with 200 µl PBS and anti–mouse IgG (H + L)-Alexa Fluor 488 was added and incubated for 20 min on ice followed by two washing steps with 200 µl cold PBS. All samples were analyzed on a BD FACSCanto-II using FACSDiva v6.1.3 software and FlowJo software v9.3.1 (Tree Star Inc.).

FcγR-Binding Assay

Human FcγRIIIa-expressing CHO cells (35) were kindly provided by Falk Nimmerjahn (Department of Biology, University of Erlangen-Nürnberg, Germany). Cells were maintained in RPMI-1640 containing P/S (50 U/ml) and 10% FCS in a humidified incubator (37°C, 5% CO_2). To detect binding of anti-CD20 antibodies, cells were detached using accutase, washed with cold PBS, and 2×10^5 cells were incubated on ice for 30 min with anti-CD20 antibodies. After incubation, cells were washed twice with 200 µl cold PBS and anti-mouse IgG (H + L)-Alexa Fluor 488 was added for 20 min on ice. All samples were washed twice with 200 µl cold PBS and analyzed on a BD FACSCanto-II using FACSDiva v6.1.3 software and FlowJo software v9.3.1 (Tree Star Inc.).

Generation of Recombinant Monoclonal Anti-CD20 IgG Isotypes

The DNA encoding for the N terminal region of the light chain (amino acids QIVLS until KLEIK) and heavy chain (amino acids QVQLQ until TVSAA) of rituximab (www.drugbank.ca; accession number DB00073) was synthesized by Invitrogen GeneArt gene synthesis. The N terminal region of the light chain was cloned into the multiple cloning site (MCS) of Igκ-AbVec (NCBI GenBank accession number FJ475056.1) using *Age*I and *Bsi*WI as previously described (36). To obtain human IgG-1, -2, -3 and -4 heavy chain expression vectors IgG-AbVec (NCBI GenBank accession number FJ475055.1) (36) was modified to encode for human IgG1 (allotype G1m17,1), IgG2 (allotype G2m), IgG3 (allotype G3m), and IgG4 (kindly provided by Lars Hangartner, the Scripps Research Institute, Department of Immunology and Microbial Science, La Jolla, CA, USA) (2, 37, 38). The N terminal region of the rituximab heavy chain was cloned into the MCS using *Age*I and *Sal*I as previously described (36). Recombinant monoclonal antibodies were expressed and purified as previously described (10, 32, 39). Briefly, heavy and light chain expression plasmids were cotransfected into a human B cell–epithelial cell fusion cell line (HKB11) supporting high-level recombinant protein expression (40) using calcium phosphate-mediated transfection. After 6 days, antibodies were purified from cell culture supernatants by binding to a protein G column and elution with 0.1 M glycine (pH 2) followed by neutralization with 1 M Tris pH 8.8 and dialysis to PBS. Antibody purity and integrity were confirmed by polyacrylamide gel electrophoresis, coomassie brilliant blue staining and Western blotting with isotype-specific monoclonal antibodies.

Generation of Galactosylated and Degalactosylated Antibody Glycovariants

Antibody glycovariants were generated as previously described (10, 39). For removal of galactose, antibodies were dialyzed to 50 mM sodium phosphate buffer pH 6.0, 60 mU β1–4 galactosidase was added per mg of antibody and the reaction was incubated for 6 h at room temperature followed by 1 h at 37°C. To add galactose, antibodies were dialyzed to 0.2 M MES pH 6.5 followed by the addition of 10 mM $MnCl_2$, UDP-galactose, 0.02% NaN_3, and 5 µg recombinant β1–4 galactosyltransferase [produced in-house (39)]. The reaction was incubated at 37°C for 48 h. Finally, all antibodies were centrifuged for 2 h at 4°C and >20,000 × *g* to remove aggregates, repurified by gravity-flow protein-G sepharose columns, and dialyzed to PBS. Successful degalactosylation and galactosylation were monitored by lectin-blotting using the galactose-specific lectin from *Erythrina Cristagalli.*

Statistics

A *p*-value of 0.05 or less was defined as statistically significant. For all analyses, Prism software, version 5 (GraphPad Software, Inc.) was used. Specific statistical tests are indicated in the respective figure legends.

AUTHOR CONTRIBUTIONS

IQ and JL conceived the study. JL designed, BP, CK, and IQ designed and performed experiments. PW performed experiments. All the authors cowrote and approved the manuscript.

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PERSPECTIVE
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Potential of Murine IgG1 and Human IgG4 to Inhibit the Classical Complement and Fcγ Receptor Activation Pathways

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IgG antibodies (Abs) mediate their effector functions through the interaction with Fcγ receptors (FcγRs) and the complement factors. The main IgG-mediated complement activation pathway is induced through the binding of complement C1q to IgG Abs. This interaction is dependent on antigen-dependent hexamer formation of human IgG1 and IgG3 to increase the affinity for the six-headed C1q molecule. By contrast, human IgG4 fails to bind to C1q. Instead, it has been suggested that human IgG4 can block IgG1 and IgG3 hexamerization required for their binding to C1q and activating the complement. Here, we show that murine IgG1, which functionally resembles human IgG4 by not interacting with C1q, inhibits the binding of IgG2a, IgG2b, and IgG3 to C1q *in vitro*, and suppresses IgG2a-mediated complement activation in a hemolytic assay in an antigen-dependent and IgG subclass-specific manner. From this perspective, we discuss the potential of murine IgG1 and human IgG4 to block the complement activation as well as suppressive effects of sialylated IgG subclass Abs on FcγR-mediated immune cell activation. Accumulating evidence suggests that both mechanisms seem to be responsible for preventing uncontrolled IgG (auto)Ab-induced inflammation in mice and humans. Distinct IgG subclass distributions and functionally opposite IgG Fc glycosylation patterns might explain different outcomes of IgG-mediated immune responses and provide new therapeutic options through the induction, enrichment, or application of antigen-specific sialylated human IgG4 to prevent complement and FcγR activation as well.

Keywords: complement, C1q, IgG4, IgG, IgG hexamer, IgG glycosylation, immunosuppression, murine IgG1

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INTRODUCTION

IgG antibodies (Abs) mediate their effector functions through the interaction with Fcγ receptors (FcγRs) and the complement system. The different IgG subclasses thereby differ by their specificities and affinities to the classical activating and inhibitory FcγRs (1–5). Furthermore, the effector function of IgG Abs depends on the type of IgG Fc glycosylation pattern. Non-(a)galactosylated IgG

Abbreviations: Ab, antibody; BCR, B cell receptor; BDCA-2, blood dendritic cell antigen 2; DC-SIGN, dendritic cell-specific ICAM-3-grabbing non-integrin; DCIR, dendritic cell inhibitory receptor; IC, immune complex; FcγR, Fcγ receptor; OVA, ovalbumin; RBC, red blood cell; sv, swich variant; SIGN-R1, specific ICAM-3-grabbing non-integrin-related 1; TNBS, 2,4,6-trinitrobenzenesulfonic acid or picrylsulfonic acid; TNP, 2,4,6-trinitrophenyl.

Abs are associated with pro-inflammatory functions, such as IgG autoAbs in rheumatoid arthritis patients, whereas galactosylated and sialylated IgG Abs have reduced inflammatory and even immunosuppressive potential (6–19). IgG Fc sialylation reduces the affinity to the classical FcγRs and, instead, promotes interaction with members of the sugar-binding C-type lectin receptor family (6–8, 13, 14, 19).

In the context of the complement activation, IgG Abs of different subclasses show highly diverse affinities to the complement C1q molecule, which initiates the classical complement pathway (3, 20–23).

Antigen-dependent hexamer formation of six monomeric human IgG1 or IgG3 Abs through non-covalent Fc:Fc interactions, such as polymeric IgM, favors the interaction with one six-headed C1q molecule leading to complement activation (22–27). Upon hexamerization, only one Fab arm of each IgG Ab interacts with the antigen. The other Fab arm is positioned in the plane of the Fc hexamer (22, 23).

By contrast, human IgG4 fails to activate the complement through C1q binding (22, 25). It has been suggested that human IgG4 might still be able to form hexamers, but subclass-specific sequence differences affect orientation of the loops in the CH2 domain, which in turn alters the position of the C1q-binding site and therewith prevents the interaction with C1q (3, 28).

On the other hand, human IgG4 as well as IgA might prevent the hexamer formation of human IgG1 and IgG3 and ultimately C1q binding by steric interference (25, 28–32).

To explore the extent of this suggested inhibition, we studied whether murine IgG1, which functionally resembles human IgG4 by not interacting with C1q, can prevent the interaction of the murine C1q-binding IgG2a, IgG2b, and IgG3 subclasses with C1q and therefore block the complement activation.

We further discuss the potential of murine IgG1 and human IgG4 to inhibit the complement activation as well as how different IgG Fc glycosylation patterns and IgG subclasses affect IgG Fc receptor interactions during IgG-mediated immune responses, which may be exploited for development of novel therapeutic strategies.

MATERIALS AND METHODS

Reagents

For the experiments described below, 2,4,6-trinitrophenyl (TNP)-coupled Ficoll (TNP-Ficoll) was purchased from Biosearch Technologies (Petaluma, CA, USA). 2,4,6-trinitrobenzenesulfonic acid or picrylsulfonic acid (TNBS)-solution was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Production of Monoclonal IgG Abs

The TNP-specific monoclonal hybridoma IgG1 (clone H5) (10, 11, 19, 33, 34) and IgG1, IgG2a, and IgG2b class-switch variant (sv; with identical VDJ sequences) (19) Abs as well as the ovalbumin (OVA)-specific IgG1 (clone 4C9) (10) Abs were produced, purified and verified as described (19).

C1q ELISA

96-well ELISA plates were coated with 10 µg of TNP-Ficoll per milliliter overnight and subsequently incubated with the indicated combinations of monoclonal anti-TNP IgG subclass Abs in PBS containing 0.1% TWEEN 20 (Sigma-Aldrich). Unbound Abs were removed with PBS containing 0.05% TWEEN 20, and wells were incubated with human serum from healthy voluntary donors diluted 1:20 in HBSS with Ca^{2+} and Mg^{2+} (Gibco® by LifeTechnologies, Paisley, UK) containing 0.1% TWEEN 20 for 1 h at room temperature. Unbound serum C1q was removed with PBS containing 0.05% TWEEN 20, and bound C1q was detected with HRP-labeled polyclonal sheep anti-human C1q IgG Abs (AbD Serotec, Bio-Rad, UK) and TMB substrate (BD Bioscience San Diego, CA, USA) at OD 450 nm. As a negative control, unspecific serum C1q binding was detected in TNP-Ficoll-coated wells without anti-TNP IgG Abs (OD at 450 nm was below 0.09).

Preparation of TNP-Labeled Red Blood Cells (RBCs)

2,4,6-Trinitrophenyl labeling of human RBCs from healthy voluntary donors was performed in cacodylate buffer (pH 6.9) containing 0.4% TNBS-solution (both from Sigma-Aldrich) for 1 h at room temperature. Subsequently, the cells were centrifuged, resuspended in PBS containing 1 mg/ml of glycylglycine (Sigma-Aldrich) and washed with PBS. TNP-coupled RBCs were stabilized in citrate-phosphate-dextrose solution containing adenine (Sigma-Aldrich) and stored up to 4 weeks at 4°C. TNP-coupling to RBCs was verified by flow cytometry with murine anti-TNP IgG2a (sv) and FITC-labeled anti-murine IgG2a (Bethyl Laboratories, Montgomery, TE, USA) Abs (Figure S1E in Supplementary Material).

Hemolysis Assay

2,4,6-Trinitrophenyl-coupled RBC suspensions (10^9 cells/ml) were prepared as described (35). 50 µl of cells was sensitized with the indicated concentrations and combinations of one or two monoclonal anti-TNP IgG subclass Abs for 30 min at 37°C. To avoid unwanted agglutination, the highest non-agglutinating concentration was determined (22.5 µg/ml for IgG2a) (data not shown). Unbound Abs were removed by washing the samples first with GVB° and then GVB^{++} (with Ca^{2+} and Mg^{2+}) buffer (ComplementTechnology, Tyler, TX, USA). Anti-TNP IgG subclass Ab-sensitized TNP-labeled RBCs were incubated with human serum from healthy volunteers diluted 1:3 in GVB^{++} buffer to induce complement-mediated RBC lysis. Complement activation and RBC lysis were stopped after 30 min at 37°C by adding 1 ml of ice cold 0.9% NaCl. Samples were centrifuged at 2,000 *g* for 5 min at 4°C and free hemoglobin in the supernatant was measured at 414 nm. The degree of RBC lysis (in %) was set in relation to a positive control lysis with H_2O (100%). As a negative control, non-IgG sensitized TNP-coupled RBCs were incubated with the same serum-GVB^{++} preparation resulting in less than 4% lysis compared with the H_2O-induced lysis. The induction of the classical complement pathway in this lysis assay was verified by blocking Ca^{2+} with EGTA (data not shown).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software. Student's *t*-test was used for comparing two groups or one-way ANOVA for comparing more than two groups: *P*-values

below 0.05 were considered statistically significant (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). EC50 values were calculated using a non-linear regression with the variable slope model.

RESULTS

Murine IgG1 Prevents Complex Formation of IgG2a, IgG2b, and IgG3 With C1q

To investigate antigen-dependent interference between non-C1q-binding murine IgG1 and C1q-binding IgG2a, IgG2b, and IgG3 subclasses for C1q binding, we used TNP-specific murine monoclonal IgG1 (clone H5), IgG3 (clone 9A6), and IgG1, IgG2a, and IgG2b class-switch variant (sv; with identical VDJ sequences) Abs (10, 19, 33, 34, 36, 37). Incubation of plate-bound TNP-Ficoll with different concentrations of single TNP-specific IgG subclasses and serum as a source of C1q showed preferential binding of C1q to IgG2a (sv), but also to IgG2b (sv) and IgG3 (9A6). The calculated half-maximal effective concentrations (EC50) of the interpolated IgG subclass-specific C1q-binding curves in this approach were 1.1 µg/ml for IgG2a (sv), 4.3 µg/ml for IgG2b (sv), and 12.5–12.9 µg/ml for IgG3 (9A6), although the EC50 value for IgG3 could not directly be compared with the values for the switch variants IgG2a and IgG2b due to differences in their VDJ sequences (**Figures 1A–C**; Figure S1 in Supplementary Material). The two monoclonal IgG1 clones (sv and H5) failed to interact with C1q in this approach (**Figures 1A–C**).

However, the combination of two different IgG subclasses showed that both IgG1 clones inhibited the binding of C1q to IgG2a, IgG2b, and IgG3 in a concentration-dependent manner (**Figures 1A–C**; Figure S1 in Supplementary Material). The IgG1 clone H5 showed a stronger inhibition than the IgG1 sv clone

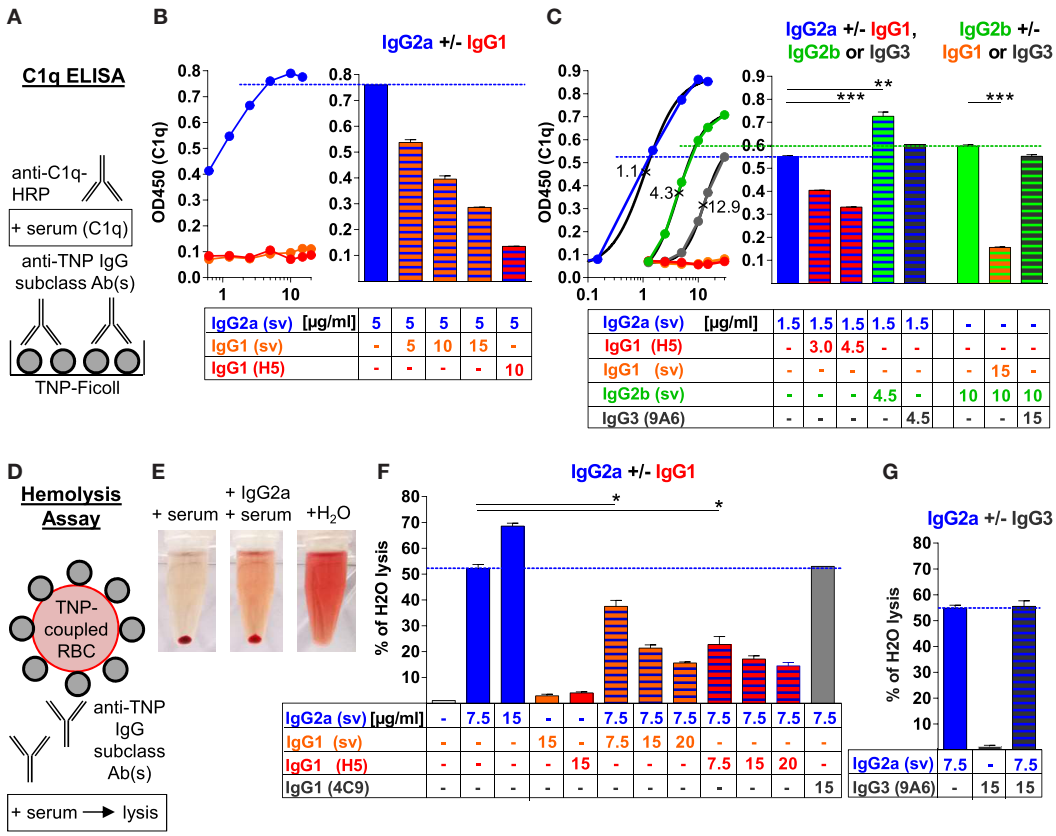

FIGURE 1 | Murine IgG1 prevents C1q binding and complement activation by IgG2a, IgG2b, or IgG3 antibodies (Abs) in an antigen-specific manner. **(A)** Schematic description of the C1q ELISA applied in panels **(B,C)** and Figure S1 in Supplementary Material. 2,4,6-Trinitrophenyl (TNP)-Ficoll-coupled 96-well plates were incubated with different concentrations of one or two anti-TNP monoclonal IgG subclass Abs [anti-TNP IgG1 (clone H5; red), IgG3 (clone 9A6; gray) as well as IgG1 (orange), IgG2a (blue), and IgG2b (green) class-switch variants (sv; with identical VDJ sequences)] and subsequently with C1q-containing serum; C1q was detected with an anti-C1q-HRP-coupled secondary Ab system. **(B,C)** Mean of the resulting C1q ELISA values measured at 450 nm (OD450) with the indicated (µg/ml) single or paired anti-TNP IgG subclass Abs ($n = 2$). IgG subclass-specific half-maximal effective concentrations (EC50; ×) were calculated by the interpolated subclass-specific C1q-binding curves in panel **(C)** ($R^2 > 0.99$). **(D)** Schematic description of the applied red blood cell (RBC)-lysis assay. TNP-coupled RBCs (Figure S1E in Supplementary Material) were incubated with one or two anti-TNP monoclonal IgG subclass Abs or an anti-ovalbumin IgG1 (clone 4C9) Ab and subsequently treated with serum containing C1q and further complement components. **(E)** Exemplary hemolysis approach with centrifuged TNP-coupled RBCs after reaction with serum, serum plus anti-TNP IgG2a or H_2O as a positive control (100% lysis). **(F,G)** Mean of the resulting RBC lysis [measured hemoglobin (OD 414 nm) in the supernatant of centrifuged RBCs], which was calculated as the percentage of H_2O-induced positive control RBC lysis (100%; maximum of the y-axes) with the indicated (µg/ml) single or paired IgG subclass Abs ($n = 2$). The results from one of at least two independent experiments are presented.

because of a higher affinity to TNP (measured by affinity ELISA; data not shown).

At an almost saturated C1q-binding dosage of IgG2a (5 µg/ml), C1q binding was inhibited about 2- or 10-fold by a 2-fold amount of the IgG1 sv or using IgG1 clone H5 of higher affinity, respectively (**Figure 1B**; Figure S1A in Supplementary Material). At an IgG2a concentration (1.5 µg/ml) near its EC50, C1q binding to IgG2a was inhibited about 1.4-fold by a 2-fold amount of the IgG1 clone H5 (**Figure 1C**; Figure S1B in Supplementary Material). At an almost saturated C1q-binding dosage of IgG2b (10 µg/ml), C1q binding was downregulated about 4- or 9-fold by only 1.5-fold amount of the IgG1 sv or the IgG1 clone H5, respectively (Figure S1C in Supplementary Material). At IgG3 concentrations (12 or 16 µg/ml) near its EC50 value, C1q binding was even inhibited threefold to fourfold by adding only half the dose of the IgG1 clone H5 (Figure S1D in Supplementary Material).

In summary, the weaker was the C1q-binding potential of an IgG subclass, the more efficient was the inhibition by the IgG1 Abs and the higher was the concentration of IgG2a, IgG2b, or IgG3, the stronger was the dose-dependent C1q-binding inhibition by IgG1.

By contrast, a combination of IgG2a with IgG2b further enhanced C1q binding compared with IgG2a alone and a combination of IgG2a with IgG3 (at a concentration at which IgG3 showed almost no C1q binding) or IgG2b with IgG3 did not reduce C1q binding compared with IgG2a or IgG2b alone (**Figure 1C**; Figure S1A–D in Supplementary Material). Thus, the murine IgG1 subclass exclusively inhibited binding of C1q to murine IgG2a, IgG2b, and IgG3.

Murine IgG1 Prevents C1q-Mediated Complement Activation by IgG2a

Next, we investigated *in vitro* whether murine IgG1 can also inhibit complement-mediated lysis of RBCs induced by the other murine IgG subclasses. TNP was conjugated to purified RBCs (Figure S1E in Supplementary Material), and these cells were incubated with one murine IgG subclass or a combination of two different IgG subclasses. Serum was used as a source of C1q and further complement components to induce RBC lysis and hemoglobin release (**Figures 1D–G**). Complement-mediated RBC lysis was calculated as the percentage of complete lysis of the same amount of cells with H_2O (set 100%; the positive control) (**Figures 1E–G**).

IgG2a alone induced a dose-dependent C1q-mediated RBC lysis when applied in the concentration range between 2.5 and 22.5 µg/ml (the highest used non-agglutinating concentration) and reached up to 70% of positive control H_2O lysis (**Figures 1E–G** and data not shown). IgG2b alone poorly induced RBC lysis in the concentration range between 7.5 and 22.5 µg/ml and reached no more than 8% of positive control H_2O lysis (data not shown). Murine IgG1 and also IgG3 alone barely induced any RBC lysis in this setting (1–4% of positive control H_2O lysis), which was in the range of the negative control, when no IgG applied (**Figures 1F,G** and data not shown). Because IgG2b and IgG3 failed to induce significant RBC lysis, we further tested how IgG1 affects IgG2a-mediated RBC lysis only.

Both IgG1 clones inhibited IgG2a-induced RBC lysis. The IgG1:IgG2a ratio of 2:1 led to a 2.4- or 3-fold inhibition of the IgG2a-mediated lysis with the IgG1 sv or the IgG1 clone H5, respectively. By contrast, IgG2b enhanced IgG2a-induced RBC lysis, whereas IgG3 had no effect (**Figures 1F,G** and data not shown). Furthermore, the IgG1-mediated complement inhibition was antigen-specific, because antigen-unspecific, OVA-specific murine IgG1 failed to inhibit the IgG2a-mediated RBC lysis (**Figure 1F**).

Thus, the murine IgG1 subclass exclusively inhibited IgG2a- and C1q-dependent complement activation in the hemolysis assay, in an antigen-specific manner.

DISCUSSION

Murine IgG1 and Human IgG4 May Inhibit Hexamer Formation of the Other IgG Subclasses and Consequently Their C1q Binding by Steric Interference

Human IgG1 and IgG3 Abs form hexamers *via* non-covalent Fc:Fc interactions in an antigen-specific manner to bind C1q. Although it has not been determined whether the murine IgG2a, IgG2b, and IgG3 Ab subclasses can also form hexamers to bind C1q and activate the classical complement pathway, we showed that murine IgG1 can prevent the interaction of C1q-binding murine IgG subclasses with C1q, as suggested for human IgG4 (25). Dampening of C1q binding by murine IgG1 or human IgG4 might result from competition for antigen binding and/or from steric interference between antigen-bound murine IgG1 or human IgG4 and the other IgG subclasses to inhibit hexamer formation of the latter Abs (25) (**Figure 2A**). The potential binding of murine IgG1 or human IgG4 to an antigen in close vicinity to the C1q-binding IgG subclasses might interfere with Fc:Fc contact formation, potentially leading to reduced hexamerization, C1q binding, and complement activation (23, 25). Efficient steric interference may be dependent on antigen density and epitope specificity, as well as on affinity of murine IgG1 and human IgG4.

Human IgG4 exploits additional properties to modify immune responses, such as dynamic Fab-arm exchange, which results in formation of bispecific IgG4 Abs (38, 39), and the interaction with the Fc part of other IgG subclass Abs (40–45). Both mechanisms might also contribute to the human IgG4-mediated suppression of the hexamer formation by other IgG subclasses; but it has not been yet demonstrated for murine IgG1.

The possibility that antigen-dependent steric interference by murine IgG1 or human IgG4 prevents the hexamer formation by other IgG subclasses might suggest a scenario, in which a single murine IgG1 or human IgG4 molecule is sufficient to reduce the hexamer formation of the other IgG subclass molecules, conferring anti-inflammatory properties to murine IgG1 or human IgG4 *via* blocking the complement activation.

Anti-Inflammatory Functions of Murine IgG1 and Human IgG4 in the Context of FcγR Interactions

Fcγ receptor-mediated effector functions vary between IgG subclasses and depend on FcγR distribution on the surface of myeloid and lymphoid immune cells. Murine IgG1, as well

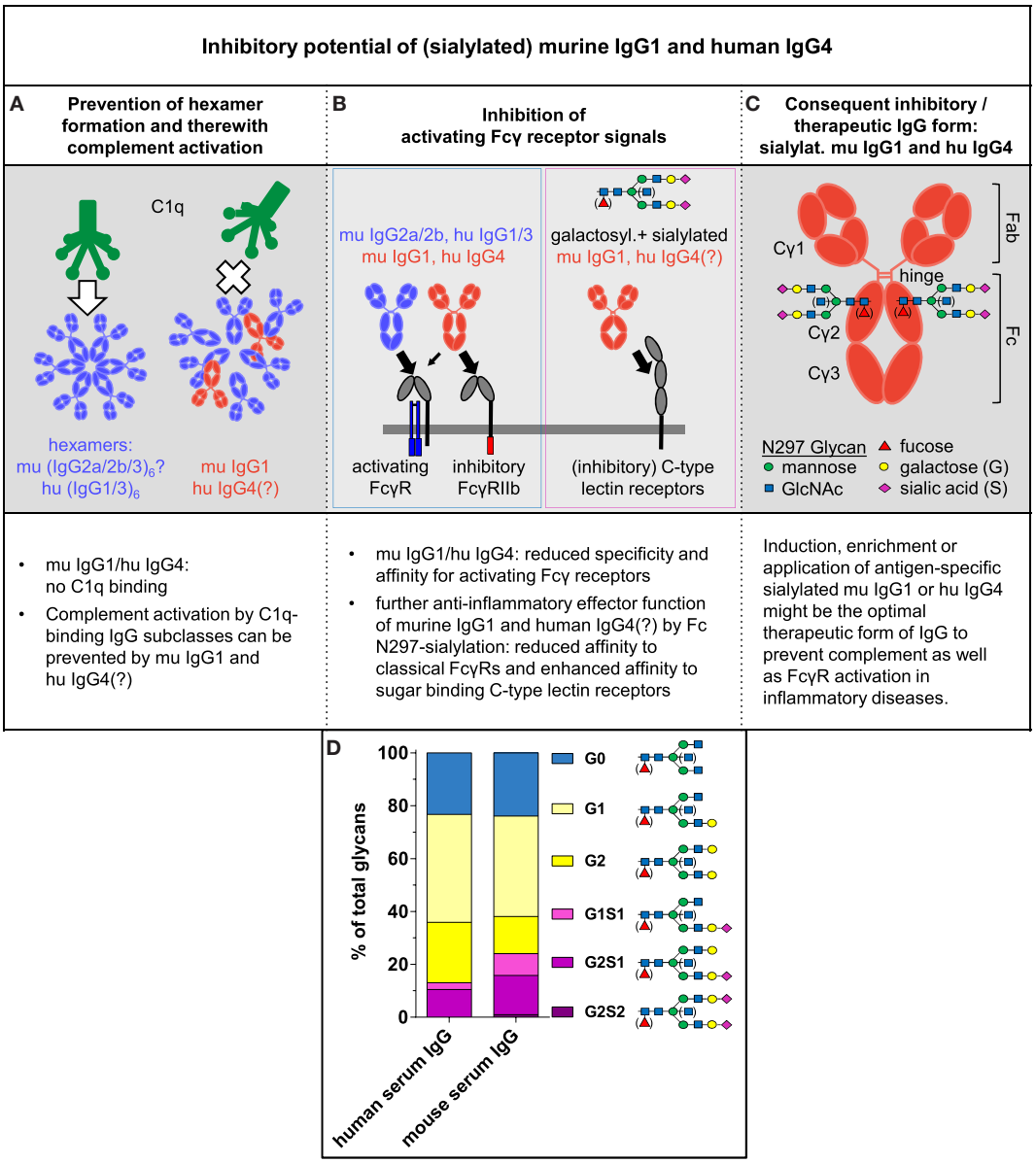

FIGURE 2 | Inhibitory potential of (sialylated) murine IgG1 and human IgG4. **(A–C)** Summary of our perspective about the inhibitory and therapeutic potential of (sialylated) murine IgG1 and human IgG4 (see text for details). IgG antibodies have one conserved *N*-glycosylation site at Asn 297 in each of their constant heavy chain regions. The biantennary core glycan structure that consists of four *N*-acetylglucosamine (GlcNAc) and three mannose residues can be further decorated with fucose, a bisecting GlcNAc and terminal galactose or galactose and sialic acid. **(D)** Average distribution of total human and mouse serum IgG Fc glycosylation patterns coupled to Asn 297 of total human serum IgG (IVIg; pooled intravenous immunoglobulin from healthy donors) and total murine serum IgG from 8- to 10-week-old untreated female C57BL/6 wild-type mice were analyzed by high-pressure liquid chromatography [data from Epp et al. (19)].

as human IgG4, show limited specificities and affinities to the classical activating FcγRs and preferably interact with the classical IgG inhibitory receptor FcγRIIb, as compared with murine IgG2a and IgG2b as well as human IgG1 and IgG3 (**Figure 2B**; Figure S2 in Supplementary Material) (1–5, 46, 47). Thus, both the IgG subclass distribution and the expression levels of classical activating and inhibitory FcγRs determine the balance between activating and inhibitory signals in the target cells.

Mechanistically, crosslinking of IgG Abs with classical activating FcγRs induces phosphorylation of their own or their associated Fc receptor gamma (FcRγ)-chain intracellular immunoreceptor tyrosine-based activation motif (ITAM) (Figure S2 in Supplementary Material) and activation of the target cell (Figure S2 in Supplementary Material) (2). However, additional IgG-mediated crosslinking of the inhibitory FcγRIIb can lead to the phosphorylation of its intracellular immunoreceptor tyrosine-based inhibition motif (ITIM) and thereby inhibition

of cell activation (Figure S2 in Supplementary Material) (2). In general, the phosphorylation of an ITIM motif always depends on upstream ITAM phosphorylation, for example, here, of activating FcγR (Figure S2 in Supplementary Material) (2).

Moreover, FcγR-mediated pro- or anti-inflammatory effector functions of murine and human IgG Abs are effected by the IgG Fc *N*-glycosylation pattern at Asn 297 in the CH2 region. Enriched amounts of non-(a)galactosylated IgG (auto)Abs are associated with pro-inflammatory effector functions of the IgG Abs and (auto)immune disease severity, such as IgG autoAbs in patients with rheumatoid arthritis, whereas galactosylated and sialylated IgG Abs possess reduced inflammatory and even immunosuppressive potential (**Figure 2D**) (6–12, 14, 17–19, 34, 48). IgG Fc galactosylation and sialylation seems to alter the conformation of the Fc portion and ultimately shifts its affinity from the classical FcγRs toward the so-called type II FcRs that mediate inhibitory signaling. Several sugar-binding C-type lectin receptors belong to the type II FcRs, including murine specific ICAM-3-grabbing non-integrin-related 1 (SIGN-R1) and its human homolog dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN); CD209, murine dendritic cell inhibitory receptor (DCIR), and CD23 (**Figure 2B**; Figure S2 in Supplementary Material) (4, 7, 8, 12–15, 18, 19, 49–53).

SIGN-R1 and DC-SIGN have originally been identified as receptors that recognize carbohydrates of some bacteria, e.g., *S. pneumonia* (4, 7, 14, 51). SIGN-R1 and DC-SIGN contain neither an ITAM nor an ITIM motif and the induction of inhibitory signals by sialylated IgG Abs *via* these receptors is yet unclear (Figure S2 in Supplementary Material).

Dendritic cell inhibitory receptor interacts with glycans of both pathogenic and endogenous origins and has, in contrast to SIGN-R1/DC-SIGN, an intracellular ITIM motif (4, 13, 49, 50, 54). However, it is still unknown whether sialylated IgG immune complexes (ICs) crosslink DCIR with ITAM-containing classical FcγRs or other ITAM-containing receptors to deliver inhibitory signals (Figure S2 in Supplementary Material).

CD23 is known as the low-affinity IgE receptor present on some immune cells, including B lymphocytes. However, also sialylated IgG Abs can crosslink CD23, which leads to up-regulation of FcγRIIB expression (Figure S2 in Supplementary Material) (4, 14, 15, 55). The induced inhibitory signals by CD23 might be mediated by crosslinking of CD23 with the B cell receptor (BCR) and thereby modulating the BCR signal.

The structure of the IgG Fc glycan has different impact on the effector functions of the different IgG subclasses: it strongly influences functional properties of murine IgG1, less of IgG2b, but barely of IgG2a (10, 11, 19, 34, 48).

Recent studies have shown that sialylated antigen-specific murine IgG1 Abs can be used to induce antigen-specific immune tolerance, making such Abs a potential therapeutic tool to treat inflammatory immune diseases in an antigen-specific manner (10–12, 16).

In addition to the anti-inflammatory potential of sialylated murine IgG1, only galactosylation of murine IgG1 has also induced inhibitory signals in a mouse model of autoimmune hemolytic anemia and *via* crosslinking the C-type lectin receptor Dectin-1 (Clec7a) with the inhibitory receptor FcγRIIb (Figure S2 in Supplementary Material) (12, 34, 56). Dectin-1 recognizes bacterial carbohydrates and activates effector cells *via* an intracellular ITAM-like (hemITAM) motif (34, 51, 57). However, in proximity to FcγRIIb, the ITAM-like motif of Dectin-1 induces phosphorylation of the ITIM motif of FcγRIIb and promotes inhibitory signaling (34). Recently, it has further been suggested that the galactose-binding receptor galectin-3 is additionally involved in this complex and directly interacts with galactosylated murine IgG1 and induces complex formation between Dectin-1 and FcγRIIb (58). In humans, inhibitory effects of galactosylated IgG Abs have been described for the plasmacytoid dendritic cell-specific type II C-type lectin receptor blood dendritic cell antigen 2, which signals *via* the ITAM-containing FcRγ-chain as signaling molecule (59, 60).

It has still to be determined to which extent Fc glycosylation affects effector properties of human IgG molecules in a subclass-dependent manner, but the current suggestion is that the effector function of all human IgG subclasses depends on the Fc glycosylation pattern.

Interestingly, the hexamer formation of human IgG1 is dependent on the conserved IgG Fc glycosylation site (23) and human IgG1 Fc galactosylation favors C1q binding, as compared with agalactosylated as well as sialylated human IgG1 (61, 62).

Together, the observations mentioned before show that sialylated murine IgG1 and seemingly human IgG4 have an anti-inflammatory potential in the context of FcγR-mediated activation of the immune system.

IgG Subclass Distribution and Fc Glycosylation Pattern Determine the Effector Function of IgG Abs, Making Sialylated Murine IgG1 and Human IgG4 Attractive Inhibitory (Therapeutic) Tools

In line with the above mentioned findings, it has recently been demonstrated that IgG1-deficient mice develop severe autoimmune conditions in different inflammatory autoimmune disease models (63, 64) as well as enhanced antigen-dependent IgG3 IC depositions in the kidneys (37). The autoimmune models used in these studies (63, 64), similar to other inflammatory autoimmune models, are apparently dependent on C1q and the complement activation as well as on classical FcγR-mediated immune cell activation (21, 61, 63–69). In future studies, it would be important to explore the inhibitory potential of murine IgG1 and its Fc glycosylation status in terms of the complement activation and FcγR-mediated regulation of effector cells using various models of autoimmunity and inflammation.

In summary, the mentioned results show that the analysis of the IgG subclass distribution and Fc glycosylation pattern during an immune response provides important information about the inflammatory state of the immune reaction. It is still a common practice that inflammatory and autoimmune diseases are diagnosed only by analysis of total (auto)antigen-specific IgG Abs. We believe that the additional analysis of IgG subclass distribution and their Fc glycosylation patterns will be essential for understanding the current inflammatory status of antigen-specific

IgG-mediated immune response and associated pathologies. These data also suggest that murine IgG1 and human IgG4 Abs in particular might have the strongest inhibitory potential toward all other IgG forms, due to their possible inhibitory effect on both, complement and classical FcγR activation (**Figure 2C**).

Total IgG fraction in human serum mainly consists of human IgG1 and less of human IgG4 (IgG1 > IgG2 > IgG3 > IgG4). Changes in this distribution of IgG subclasses as well as in their Fc glycosylation patterns will presumably have a strong impact on IgG-mediated immune responses.

For many years, the induction of human IgG4 (for instance, induced by allergen-specific immunotherapies) has been associated with the inhibition of IgE-mediated allergic immune responses (19, 70). However, in the presence of high doses of allergen IgG Abs can also induce allergic reactions (19). In this context, an inhibitory role of human IgG4 has been discussed (19, 29). Furthermore, the MHC-specific human IgG1/3 to IgG4 ratio might be a critical parameter that determines the risk of complement-mediated transplant rejection in patients after organ transplantation and blood transfusion (71). More in-depth analysis of the IgG subclass distribution and Fc glycosylation might clarify inhibitory effects of human IgG4 in these studies.

Blockade of C1q with designer molecules has been proposed as a potent approach to treat inflammatory diseases. However, the shift of antigen-specific T and B cell responses to produce primarily sialylated human IgG4 or the application of enriched or *in vitro*-produced antigen-specific sialylated IgG4 Abs might be a promising alternative therapeutic approach to treat complement-mediated inflammatory diseases and to inhibit FcγR-mediated inflammatory responses at the same time. Recent studies have indeed shown for the first time that enrichment of type XVII collagen (Col17)-specific human IgG4 autoAbs from autoimmune patients with bullous pemphigoid skin disease and their administration to humanized Col17 mice inhibited the complement activation and disease development by interfering with the other IgG subclasses (72).

CONCLUSION AND PERSPECTIVE

We showed that murine IgG1 inhibits C1q binding of IgG2a, IgG2b, and IgG3 and complement activation by murine IgG2a in an antigen-dependent manner, and suggested potential application of these findings for human IgG4. We further outline other inhibitory functions of murine IgG1 and human IgG4 and stressed the importance of future analysis of IgG subclass distribution and Fc glycosylation patterns in developing novel therapeutic approaches. We conclude that antigen-specific sialylated murine IgG1 and human IgG4 Abs might be a promising therapeutic tool to inhibit complement activation and abolish FcγR-mediated inflammatory responses in inflammatory (autoimmune) diseases.

ETHICS STATEMENT

Human blood from healthy donors was collected with the approval of and in accordance with regulatory guidelines and ethical standards set by the University of Lübeck.

AUTHOR CONTRIBUTIONS

G-ML conducted the experiments. ME supervised the experiments and discussed and wrote the manuscript together with G-ML, JR, JP, YB, and AL.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00958/full#supplementary-material.

FIGURE S1 | Inhibition of C1q binding to IgG2a, IgG2b, and IgG3 by IgG1. **(A–D)** C1q ELISA experiments as described and partially shown in **Figures 1A–C** with 2,4,6-trinitrophenyl (TNP)-Ficoll-coated 96-well plates, which were incubated with different concentrations of one or two anti-TNP monoclonal IgG subclass Abs [anti-TNP IgG1 (clone H5; red), IgG3 (clone 9A6; gray) as well as IgG1 (orange), IgG2a (blue), and IgG2b (green) class-switch variants (sv; with identical VDJ sequences)] and subsequently with serum containing C1q that was detected with an anti-C1q-HRP-coupled secondary Ab system. The left figure parts in panels **(A–D)** show the single IgG2a (blue), IgG2b (green), or IgG3 (gray) OD 450 nm (OD 450; left *y*-axes; each point: mean of $n = 2$) value binding curves and their interpolated binding curves with the percentage (right *y*-axes) of their maximal C1q binding [black; R^2 of interpolation was >0.99; the top of the (interpolated) IgG subclass-specific curves was set to 100%, and the bottom of the curves was set to 0%]. The IgG subclass-specific half-maximal effective concentration (EC50) was calculated from the interpolated C1q-binding curves. To the indicated (dashed line) amounts of single IgG subclasses [**(A)** 5 µg/ml IgG2a; **(B)** 1.5 µg/ml IgG2a; **(C)** 10 µg/ml IgG2b; **(D)** 12 or 16 µg/ml IgG3], the indicated *x*-fold amounts of a second indicated anti-TNP IgG subclass were added. Their resulting OD 450 values and calculated percentages are presented in the right figure parts. **(E)** Verification of the TNP-coupling to red blood cell (RBC) as described in **Figure 1D**. TNP coupling to RBCs was verified by flow cytometry with murine anti-TNP IgG2a (sv) and FITC-labeled anti-murine IgG2a Abs. The results from one of at least two independent experiments are presented.

FIGURE S2 | Expression of IgG Fc interacting receptors by human and murine immune effector cells and their immunoreceptor tyrosine-based activation motif (ITAM)/immunoreceptor tyrosine-based inhibition motif (ITIM) signaling motifs. List of type I and type II IgG Fc receptors as mentioned in the text. The expression pattern of the individual receptors on different immune cell types has been described before (1–5, 7, 8, 12–15, 18, 19, 34, 46, 47, 49–54, 57–60). Abbreviations: BC, B cells; TC, T cells; NK, natural killer cells; Mo, monocytes; MΦ, macrophages.

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Binding of von Willebrand Factor to Complement C1q Decreases the Phagocytosis of Cholesterol Crystals and Subsequent IL-1 Secretion in Macrophages

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Complement C1q, the initiation molecule of the classical pathway, exerts various immunomodulatory functions independent of complement activation. Non-classical functions of C1q include the clearance of apoptotic cells and cholesterol crystals (CC), as well as the modulation of cytokine secretion by immune cells such as macrophages. Moreover, C1q has been shown to act as a binding partner for von Willebrand factor (vWF), initiation molecule of primary hemostasis. However, the consequences of this C1q-vWF interaction on the phagocytosis of CC by macrophages has remained elusive until now. Here, we used CC-C1q-vWF complexes to study immunological effects on human monocyte-derived macrophages (HMDMs). HMDMs were investigated by analyzing surface receptor expression, phagocytosis of CC complexes, cytokine secretion, and caspase-1 activity. We found that vWF only bound to CC in a C1q-dependent manner. Exposure of macrophages to CC-C1q-vWF complexes resulted in an upregulated expression of phagocytosis-mediating receptors MerTK, LRP-1, and SR-A1 as well as CD14, LAIR1, and PD-L1 when compared to CC-C1q without vWF, whereas phagocytosis of CC-C1q complexes was hampered in the presence of vWF. In addition, we observed a diminished caspase-1 activation and subsequent reduction in pro-inflammatory IL-1β cytokine secretion, IL-1β/IL-1RA ratio and IL-1α/IL-1RA ratio. In conclusion, our results demonstrate that vWF binding to C1q substantially modulates the effects of C1q on HMDMs. In this way, the C1q-vWF interaction might be beneficial in dampening inflammation, e.g., in the context of atherosclerosis.

Keywords: macrophages, complement C1q, von Willebrand factor, cholesterol, atherosclerosis, innate immunity

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INTRODUCTION

The complement system is a highly effective part of the innate immune system. The multiple functions of complement include defense against bacterial infections, bridging innate and adaptive immunity and the clearance of immune complexes, and components of inflammation (1). The complement system can be activated through three distinct pathways: the classical, the lectin and the alternative pathway. All three pathways converge in a shared terminal response resulting in the formation of C5a and C3a as potent inflammatory effector molecules and C5b-9

as membrane attack complex. However, each pathway is initiated through different characteristic recognition molecules (2). The initiation of the classical pathway is triggered by C1q through sensing of bound antibodies as well as pathogen- and damage-associated molecular patterns (PAMPs/DAMPs). In addition, more recent research has shown a number of functions for C1q that are independent of downstream complement activation (3). On the one hand, opsonization with C1q enhances the clearance of diverse structures, namely immune complexes (4) and apoptotic cells (5) as well as atherogenic lipoproteins (6) and cholesterol crystals (CC) (7) by phagocytes. On the other hand, anti-inflammatory properties for C1q have been well-described. For example, bound C1q decreases the release of pro-inflammatory cytokines and increases the production of anti-inflammatory mediators by phagocytes (8, 9). Additionally, the presence of C1q on apoptotic cells skews macrophage polarization toward an anti-inflammatory phenotype (10).

Apart from C1q's extensively studied involvement in immunity, a complex cross-talk between complement and coagulation is becoming more and more evident (11). Complement components have been found to induce hemostasis and *vice versa* coagulation factors can trigger complement activation, thereby combining two powerful plasma cascades. Within the hemostatic cascade, von Willebrand factor (vWF) acts as an important starter molecule by mediating platelet adhesion and aggregation. Immune cells, such as macrophages, are competent to take up and clear vWF through scavenger receptors (12, 13). Moreover, vWF has been shown to interact with complement factor H (14, 15) and therefore can modulate the activation of complement via the alternative pathway (16). Furthermore, a direct interaction between vWF and C1q was found by our group, demonstrating that C1q, bound to surfaces such as apoptotic cells, acts as a binding partner for vWF (17). The C1q-vWF interaction also seems to occur on the surface of CC.

CC can be found as a characteristic feature in the intima of atherosclerotic arteries from early lesions to late plaque (18) and are widely used in *in vitro* models of atherosclerosis (19–21). Formation of CC occurs upon fatty streak development by an increased uptake and exhausted efflux of cholesterol by lipid-laden macrophages known as foam cells. In *in vitro* and *in vivo* models of atherosclerosis, CC have been implicated in the activation of the NOD [nucleotide oligomerization domain]-, LRR [leucine-rich repeat]-, and PYD [pyrin domain]-containing protein 3 (NLRP3) inflammasome and downstream cytokine secretion, consequently triggering local and systemic inflammation (22–24). While the role of CC and macrophages in atherosclerosis appears unambiguous, C1q can play a dual role. On the one hand, C1q bound to oxidized low-density lipoproteins (LDL) or CC has been shown to activate the classical pathway, and in this context to drive the progression of atherosclerosis in animal models (25, 26). On the other hand, C1q has also been described to be protective in early atherosclerosis *in vivo* (27, 28) and to increase cholesterol efflux transporter expression *in vitro* (6), suggesting atheroprotective properties. Similarly, the role of vWF in atherosclerosis is still a matter of debate. Although various studies suggest that vWF deficiency provides protection from atherosclerosis in animals, in humans, an unequivocal protective effect of vWF deficiency on atherosclerosis has not been demonstrated so far (29).

In summary, CC, macrophages, C1q and vWF have all been implicated in atherosclerosis. Nevertheless, the consequences of the interaction between C1q and vWF, especially on phagocytes, remain to be determined. In order to better understand this interaction, the aim of our study was to investigate the immunological effect of complexes consisting of cholesterol crystals, C1q and von Willebrand factor (CC-C1q-vWF complexes) by studying receptor expression, phagocytosis and cytokine secretion of macrophages.

MATERIALS AND METHODS

Preparation of CC

Cholesterol (suitable for cell culture, Sigma Aldrich, St. Louis, MO, USA) was dissolved in 95% ethanol at 60°C (12.5 g/l), sterile filtered and allowed to crystallize at room temperature (RT) for 7 days (d). Excess liquid was removed from the suspension, followed by drying for 5 d. Finally, CC were ground and stored as stock CC at −20°C until use.

Preparation of CC, CC-C1q, and CC-C1q-vWF Complexes for Characterization of C1q and vWF Binding

Dry stock CC were weighed and suspended in PBS (Life Technology, Carlsbad, CA, USA) at a concentration of 1.6 mg/ml, vortexed, and sonicated until a visually homogenous suspension was achieved. This CC suspension was split in three fractions for generation of CC, CC-C1q complexes, and CC-C1q-vWF complexes. Fractions were washed with PBS by centrifugation (1,000 x g, 5 min, RT) and resuspended at the same concentration. For generation of CC-C1q complexes, 50 μg/ml purified C1q (Complement Technology, Tyler, Tx, USA), diluted in PBS, was added and incubated for 1 h at RT on a shaker (700 rpm). Afterwards, CC and CC-C1q complexes were washed (as described above). For generation of CC-C1q-vWF complexes, 10 μg/ml recombinant vWF {provided by Baxalta, Lexington, MA, USA [former Baxter; characterization by Turecek et al. (30)]}, diluted in PBS, was added to washed CC-C1q complexes, vortexed rigorously, and further incubated for 1 h at RT on a shaker (700 rpm). After another washing step, CC complexes were further incubated with monoclonal mouse anti-C1q [clone 32A6 cell supernatant (31)], diluted 1:20 in PBS, or polyclonal rabbit anti-vWF (Abcam, Cambridge, UK), diluted 1:1,000 in PBS, for 1 h at RT on a shaker (700 rpm). Secondary antibody staining was performed with donkey anti-mouse IgG-AlexaFluor (AF)555 (Life Technology) and goat anti-rabbit IgG-AF647 (Abcam), both diluted 1:200 in PBS/1%BSA (Sigma Aldrich)/0.5 M NaCl for 30 min at 4°C in the dark, followed by a final wash step and resuspension in PBS/1%BSA/0.5 M NaCl. All fractions were washed and treated with either active substance (protein or antibodies) or solution only in the same manner. For flow cytometry, data were acquired using a BD Accuri 6 (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo10.

For confocal microcopy, CC were spun onto cytoslides (Shandon, Pittsburg, PA, USA) by a Cytospin centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed using Nikon A1R Nala and NIS software (both Nikon, Tokyo, Japan). For imaging flow cytometry, analyses were carried out using ImageStreamX Mark II and IDEAS software (both EMD Millipore, Billerica, MA, USA).

Cell Culture

Peripheral blood mononuclear cells were isolated from fresh buffy coats (Blood Transfusion Center of the University Hospital Basel, Basel, Switzerland) by density gradient centrifugation using Lymphoprep (Stemcell Technologies, Vancouver, Canada). Monocytes were obtained by CD14+ magnetic-activated cell separation beads (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions (yielding an average purity of 95–98% CD14+ monocytes determined by flow cytometry). Monocytes were differentiated into human monocyte-derived macrophages (HMDMs), cultured in DMEM supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (DMEM+), 10% fetal calf serum (FCS) (all from Life Technology), and 50 ng/ml GM-CSF (Immunotools, Frisoythe, Germany) at a cell concentration of 5×10^5 cells/ml in 6-well plates (BD Biosciences, Franklin Lakes, NJ, USA) and maintained in 5% CO_2 at 37°C for 7 days.

Treatment With CC Complexes

After 7 days, HMDMs were washed with prewarmed DMEM+, optionally stimulated with 100 ng/ml lipopolysaccharide (LPS) (*E. coli* O127:B8, Sigma Aldrich), diluted in prewarmed DMEM+, and treated with CC, CC-C1q, or CC-C1q-vWF complexes for indicated time points. CC and CC-complexes were prepared as described above, washed once with PBS by centrifugation (1,000 × g, 5 min, RT) and resuspended in prewarmed DMEM+ at a final concentration of 0.5 mg/ml before adding to cells.

Surface Receptor Expression

HMDMs were stimulated with LPS and treated with CC, CC-C1q, or CC-C1q-vWF complexes as described above. After 18 h, HMDMs were washed with PBS and incubated with PBS/10 mM EDTA (AppliChem, Darmstadt, Germany) for 30 min at 4°C. Cells were collected in FACS buffer (PBS/0.1% FCS/1 mM EDTA) and resuspended at a cell concentration of 5×10^5 cells/100 μl and incubated with 2 μg/ml of human IgG for 45 min at 4°C to block unspecific binding of antibodies to Fcγ receptors. Staining was performed for 30 min at 4°C in the dark in PBS using the following antibodies: anti-MHC II-FITC (Immunotools), anti-tyrosine-protein-kinase Mer (MerTK)-PE (R&D Systems, Minneapolis, MN, USA), anti-programmed death ligand 1 (PD-L1/CD274)-APC and anti-CD14-PeCy7 (both from Biolegend, San Diego, CA, USA) (antibody panel 1), or anti-CD86-FITC (Biolegend), anti-lipoprotein receptor-related protein 1 (LRP-1/CD91)-PE (Thermo Fisher Scientific, Waltham, MA, USA), anti-leukocyte-associated immunoglobulin-like receptor 1 (LAIR1/CD305)-AF647 and anti-scavenger receptor A 1 (SR-A1/CD204)-PeCy7 (both from Biolegend) (antibody panel 2). HMDMs were washed and resuspended in FACS buffer. Data were aquired using a BD LSRFortessa (BD Biosciences) and analyzed with FlowJo10. Gating was performed on SSC/CD14+ cells (antibody panel 1) or SSC/CD91+ cells (antibody panel 2), respectively, and geometric mean fluorescence intensity (gMFI) was calculated.

Phagocytosis Assay

Assessment of Granularity of HMDMs

HMDMs were kept untreated or treated with CC, CC-C1q or CC-C1q-vWF complexes as described above. After 18 h, HMDMs were harvested with PBS/10 mM EDTA and resuspended at a cell concentration of 5×10^5 cells/100 μl in FACS buffer. HMDMs were stained with anti-CD11c-APC (Biolegend) for 30 min at 4°C in the dark. Data were aquired using a BD LSRFortessa (BD Biosciences) and analyzed with FlowJo10. For the quantification of phagocytosis the percentage of CD11c+ cells with high cell granularity, indicated by a shift into the side scatter (SSC) ^{high} gate (gate set according to shift in SSC from CD11c+ untreated to CC treated cells), was determined.

Assessment of Phagocytosed pHrodo-Dyed CC Complexes

HMDMs were harvested with PBS/10 mM EDTA and resuspended in phagocytosis buffer (DMEM+/12.5 mM HEPES (Sigma Aldrich)/5 mM $MgCl_2$) at a density of 5×10^5 cells/100 μl. For pHrodo-dyed CC complexes, 1 mg/ml CC were suspended in 0.1 M $NaHCO_3$ buffer (pH 8.3) and incubated with 10 μg/ml pHrodo Red Ester (Thermo Fisher Scientific) for 1 h in the dark before the addition of C1q or C1q-vWF as described above. After a final wash, pHrodo-dyed CC complexes were added to HMDMs at a concentration of 0.5 mg/ml and incubated at 37°C for 30 min. Unphagocytosed CC complexes were washed away and HMDMs were stained with anti-CD11c-FITC (Bio-Rad, Hercules, CA, USA) for 30 min at 4°C in the dark and resuspended in FACS buffer. Data were aquired using a Beckman Coulter CytoFLEX (Beckman Coulter, Brea, CA, USA) and analyzed with FlowJo10. For the quantification of phagocytosis, the percentage of CD11c+ cells with a shift into the pHrodo Red Ester+ gate (gate set according to shift in pHrodo Red Ester from CD11c+ untreated to CC treated cells) was determined.

Quantification of Secreted Cytokine Levels

HMDMs were stimulated with LPS and treated with CC, CC-C1q, or CC-C1q-vWF complexes as described above. After 18 h, supernatants were collected, centrifuged to remove cellular debris and CC and stored at −80°C until measurement. Analyses of cytokine secretion were carried out in duplicates with ELISA kits according to the manufacturer's instructions. IL-1ß, IL-1α, IL-6, and IL-10 were measured using Biolegend ELISA kits, IL-18 and IL-1RA using Abcam ELISA kits and TNFα using a BD Bioscience ELISA kit.

Caspase-1 Activity Assay

HMDMs were kept untreated or treated with CC, CC-C1q, or CC-C1q-vWF complexes as described above. After 18 h, HMDMs were harvested with PBS/10 mM EDTA and

resuspended at a cell concentration of 5×10^5 cells/ml. Cells were incubated for 1 h with fluorochrome-labeled inhibitors of caspases (FLICA) probes for caspase-1 detection according to the manufacturer's instruction (FAM FLICA Caspase-1 Assay Kit, Immunochemistry Technology, Bloomington, MN, USA). HMDMs were stained with anti-CD11c-APC (Biolegend) for 30 min at 4°C in the dark. Data were aquired using a BD LSRFortessa (BD Biosciences) and analyzed with FlowJo10. Quantification of caspase-1 activity was determined by the percentage of CD11c+ cells in the FLICA+ gate.

Statistical Analysis

Data are expressed as median ± interquartile range (IQR), if not stated otherwise. Wilcoxon matched pairs signed rank test was used to compare two groups of paired data. When more than 2 groups of unpaired data were compared, Kruskal-Wallis test was performed and if significant followed by Mann-Whitney *U*-test for comparison of two specified groups as indicated. Data were analyzed with a statistical package program (GraphPad Prism 8, La Jolla, CA, USA). Differences were considered statistically significant when the $p < 0.05$.

RESULTS

vWF Binds to CC in a C1q-Dependent Manner

Whereas, C1q is described as a classical opsonin for a variety of DAMPs (10), the molecule has been also shown to adhere to oxidized LDL (6). In addition, Samstad et al. demonstrated C1q binding on CC after incubation with human plasma (7). Therefore, we first analyzed whether surface-bound C1q on CC secondarily enables the binding of vWF. We characterized the binding of vWF to C1q on the surface of CC by flow cytometry (**Figures 1A–C**), confocal microscopy (**Figure 1D**), and imaging flow cytometry (**Figure 1E**). C1q deposition on the surface of CC is shown in **Figure 1A**. The incubation of CC with vWF in the absence of C1q showed no vWF deposition on the CC surface (orange histogram in **Figures 1B,C**). Only in the presence of surface-bound C1q, vWF was enabled to bind (green histogram in **Figures 1B,C**). The gMFI for vWF binding in the presence of C1q was 50-fold higher compared to CC without C1q [median gMFI (IQR) of C1q+vWF: 115,000 (102,000–175,000) vs. vWF: 2,300 (1,500–2,400), $p = 0.0079$]. Furthermore, we analyzed the localization of vWF binding to CC-C1q complex. Using confocal microscopy, C1q and vWF could be visualized on CC. C1q and vWF stainings co-localized (**Figure 1D**). Finally, we used imaging flow cytometry to analyze a larger CC population as CC have a heterogenous structure. Again, we observed a similar staining pattern for C1q and vWF on CC (**Figure 1E**).

Taken together, our results demonstrate that bound C1q mediates the binding of vWF to CC, and vWF alone is not able to bind to the surface of CC.

CC-C1q-vWF Complexes Upregulate the Surface Receptor Expression of HMDMs

Macrophages have a high degree of plasticity, enabling these cells to change their phenotype according to the environmental stimuli (32). In this context, C1q has been shown to elicit upregulated expression of MerTK receptor, which is involved in the process of dead cell removal, termed efferocytosis (33). Moreover, it has been described that stimulation of macrophages with C1q leads to a polarization of these cells toward an anti-inflammatory state (34). Therefore, we aimed to investigate the phenotype of HMDMs in our *in vitro* model. For this purpose, HMDMs were kept untreated or treated with CC, CC-C1q, or CC-C1q-vWF complexes for 18 h. To mimic the inflammatory milieu present in, e.g., atherosclerotic plaques (35), HMDMs were simultaneously exposed to 100 ng/ml LPS for 18 h. The phenotype was studied by analyzing the expression of surface CD14, CD86, LAIR1, LRP-1, MerTK, MHC II, PD-L1, and SR-A1 (**Figures 2A–H**). HMDMs treated with CC-C1q-vWF complexes significantly upregulated the expression of CD14 ($p = 0.0312$), LAIR1 ($p = 0.0312$), LRP-1 ($p = 0.0312$), MerTK ($p = 0.0312$), PD-L1 ($p = 0.0312$), and SR-A1 ($p = 0.0312$) as compared to CC-C1q complexes without vWF. In four out of six donors, CD86 expression was upregulated, while MHC II expression was downregulated in five out of six donors. Neither the median receptor expression of CD86 nor of MHC II was significantly affected. Also, CC treatment did not induce any significant changes in surface receptor expression as compared to untreated HMDMs (data not shown).

Our results demonstrate that CC-C1q-vWF complexes uniquely affect the expression of surface receptors, namely an upregulation of efferocytosis receptor MerTK, scavenger receptors LRP-1 and SR-A1 as well as CD14, LAIR1, and PD-L1.

Phagocytosis of CC-C1q-vWF Complexes by HMDMs Is Hampered

Since C1q is involved in the processes of efferocytosis (36) as well as phagocytosis (9) and as the additional presence of vWF upregulates efferocytosis and scavenger receptors (**Figure 2**), we next investigated the role of C1q-vWF binding in the uptake of CC complexes by HMDMs (**Figure 3**). Therefore, HMDMs were kept untreated or treated with CC, CC-C1q, or CC-C1q-vWF complexes for 18 h. The phagocytosis of CC led to an increase in cell granularity, which could be determined by a shift in SSC using flow cytometry. Analyzed as control, untreated CD11c+ HMDMs did not express a SSC^{high} population. When HDMDs were treated with CC, CC-C1q, or CC-C1-vWF complexes, the cells exhibited a SSC^{high} population (**Figure 3A**). HMDMs showed a significant decrease in cells positive for phagocytosis after the treatment with CC-C1q-vWF complexes compared to CC-C1q complexes [median phagocytosis (IQR) in six independent donors of CC-C1q-vWF: 13.65% (5.83–16.35%) vs. CC-C1q: 24.05% (22.55–34.60%), $p = 0.0312$] (**Figure 3B**). To exploit the effect on early phagocytosis, we incubated CC with the pH-dependent pHrodo Red dye (**Figures 3C,D**). Analyzed as control, unstimulated CD11c+ HMDMs only exhibited a dim fluorescent signal for pHrodo Red. Fluorescent signal for pHrodo Red increased strongly when HMDMs were treated with pHrodo-dyed CC complexes for 30 min, due to the fusion of phagocytosed CC with the acidic lysosome of HMDMs. For the early phagocytosis, HMDMs had phagocytosed significantly

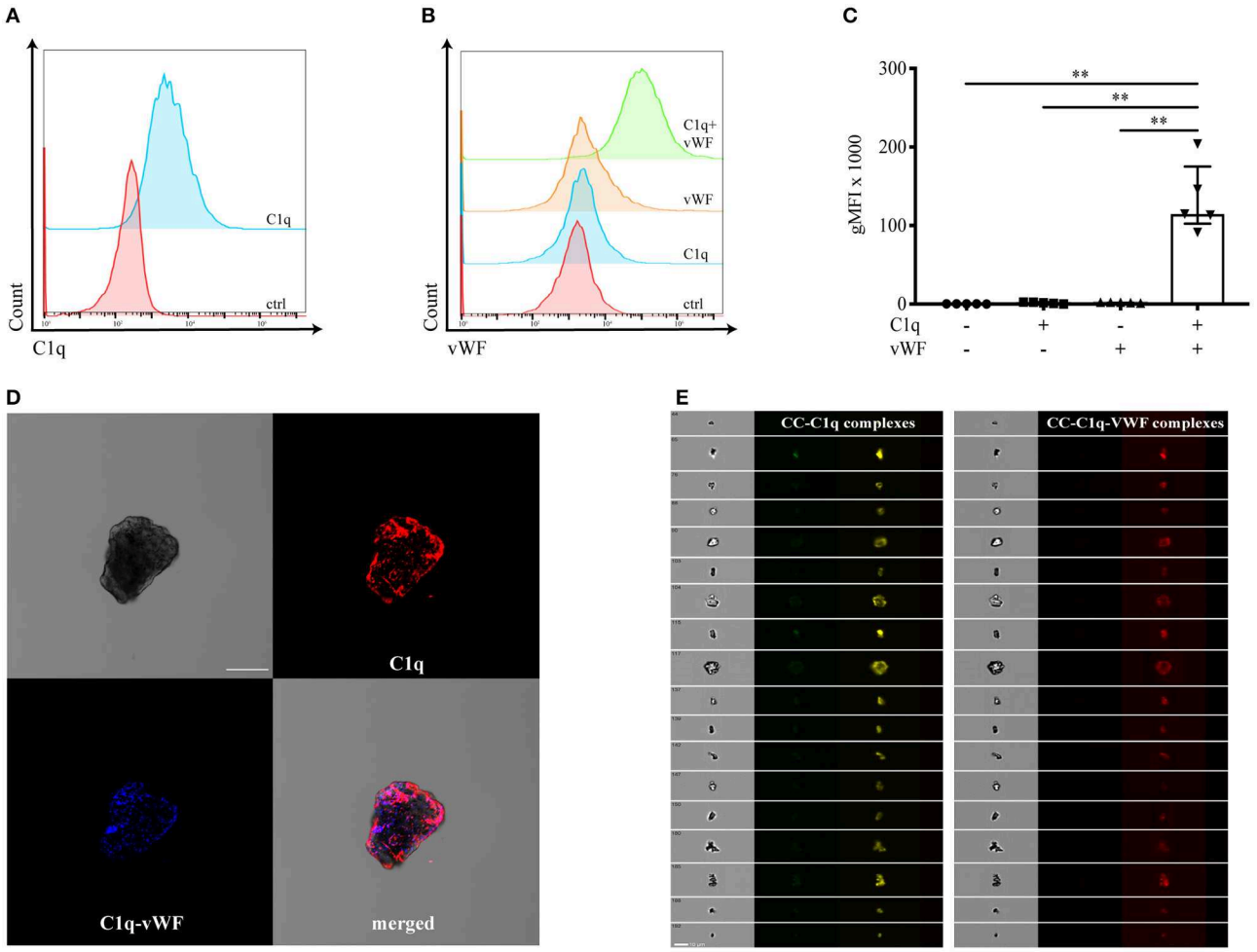

FIGURE 1 | vWF binds to CC in a C1q-dependent manner. Binding of C1q and vWF to surface-bound C1q on CC was determined by **(A–C)** flow cytometry, **(D)** confocal microscopy, and **(E)** ImageStreamX. Representative flow cytometry diagrams show the binding of **(A)** C1q or **(B)** C1q-dependent binding of vWF on the surface of CC. Controls (ctrl) represent the presence of secondary antibodies only. **(C)** Flow cytometry data are shown as median gMFI with IQR ($n = 5$, Mann-Whitney U-test, $^{**}p < 0.01$). **(D)** Confocal microscopy depicting a representative CC in brightfield, with C1q (red) or vWF (blue) in the presence of C1q bound to its surface. The merged staining patterns visualize the C1q-vWF interaction. One of three independent experiments is shown. Scale bar = 50 μm. **(E)** Fluorescent staining of CC captured by ImageStreamX for C1q (yellow) or C1q-vWF (red). One of two independent experiments is shown. Scale bar = 10 μm.

less CC-C1q-vWF complexes than CC-C1q complexes [median phagocytosis (IQR) in six independent donors of CC-C1q-vWF: 54.55% (40.05–60.03%) vs. CC-C1q: 62, 40% (49.05–68.78%), $p = 0.0312$] (**Figure 3D**).

In summary, late as well as early phagocytosis, by HMDMs, of CC-C1q-vWF complexes is reduced as compared to CC-C1q complexes.

CC-C1q-vWF Complexes Reduce IL-1 Cytokine Secretion of HMDMs

CC have been repeatedly described as capable inducers of IL-1ß secretion in human monocytes and macrophages (23). On the contrary, C1q has been shown to dampen pro-inflammatory cytokine secretion for the same cell types (34). Consequently, we next examined the effect of CC-C1q-vWF complexes on the cytokine profile of HMDMs. For this purpose, HMDMs kept untreated or treated with CC, CC-C1q, or CC-C1q-vWF complexes were stimulated with 100 ng/ml LPS for 18 h and supernatants were analyzed for the secretion of IL-1ß, IL-1α, IL-1RA, IL-18, IL-6, IL-10, and TNFα cytokine levels (**Figure 4**). The CC treatment induced a strong IL-1ß and IL-1α secretion by HMDMs and a moderate increase in IL-18 secretion as compared to untreated HMDMs. A robust decrease in pro-inflammatory cytokines for IL-1ß and IL-1α was observed with CC-C1q complexes, and a decreasing trend for IL-6 and TNFα secretion. The additional presence of vWF on CC-C1q complexes significantly enhanced reduction of IL-1ß secretion ($p = 0.0078$), IL-1ß/IL-1RA ratio ($p = 0.0078$), and IL-1α/IL-1RA ratio ($p = 0.0234$) compared to CC-C1q complexes alone. No other cytokines were significantly changed by vWF bound to CC-C1q complexes. Differences in cytokine secretion of HMDMs according to the treatment added were not due to differences in

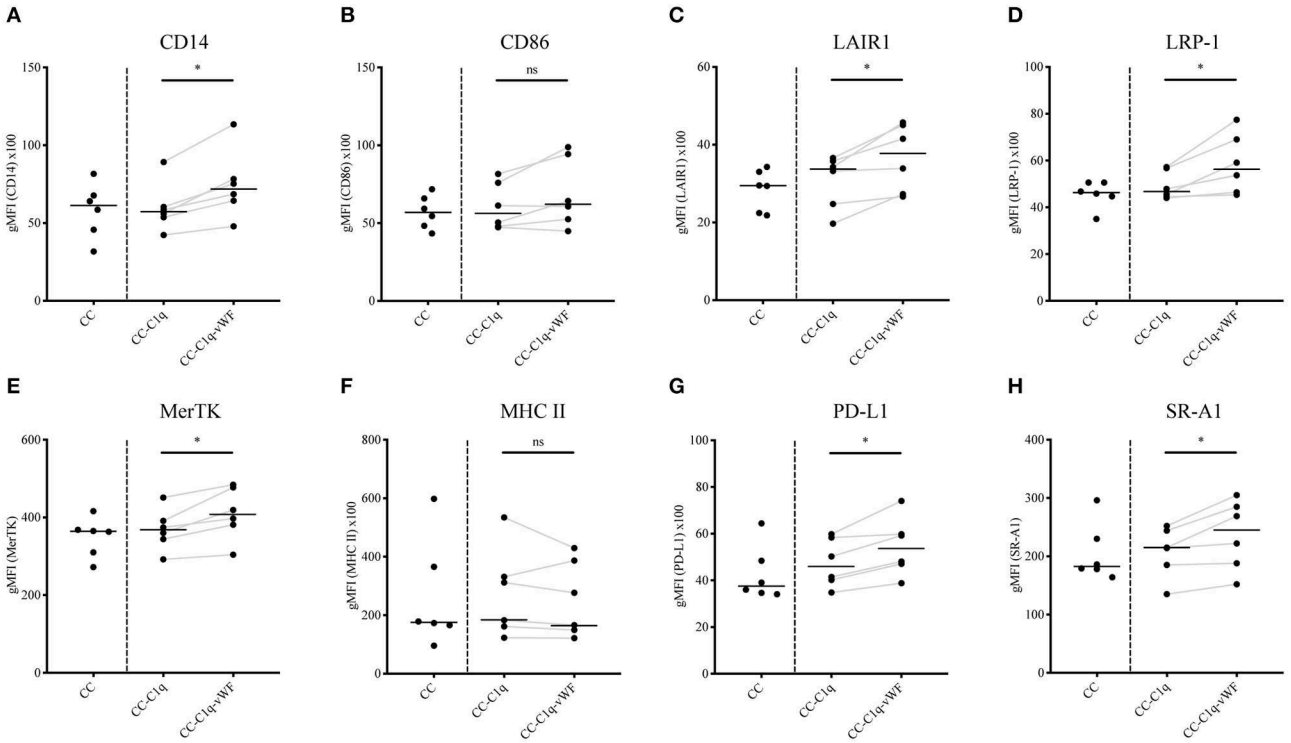

FIGURE 2 | CC-C1q-vWF complexes upregulate the surface receptor expression of HMDMs. LPS-stimulated HMDMs were treated with CC, CC-C1q or CC-C1q-vWF complexes for 18 h and analyzed by flow cytometry for surface marker expression of **(A)** CD14, **(B)** CD86, **(C)** LAIR1, **(D)** LRP-1, **(E)** MerTK, **(F)** MHC II, **(G)** PD-L1, and **(H)** SR-A1. Pooled flow cytometry data are shown as gMFI with horizontal lines denoting median. Data points represent independent experiments analyzing six different healthy donors used to obtain HMDMs (Wilcoxon matched pairs signed rank test, $*p < 0.05$; ns, not significant).

cell death as assessed by quantification of early and late apoptosis or necrosis (data not shown).

Taken together, our data show that IL-1ß cytokine secretion and IL-1ß/IL-1RA and IL-1α/IL-1RA ratio by HMDMs after exposure to CC-C1q complexes are diminished further in the presence of vWF. This reduction appears to be IL-1 specific.

CC-C1q-vWF Complexes Suppress Caspase-1 Activity of HMDMs

It is well-known that IL-1 maturation, cleavage and secretion is regulated transcriptionally as well as post-transcriptionally. While a priming signal through pattern recognition receptors is required for pro-IL-1β transcription, the maturation is dependent on the formation of the NLRP3 inflammasome and subsequent caspase-1 activation (37). Therefore, we aimed to examine whether the observed change in IL-1 cytokine secretion was the result of a preceding NLRP3 inflammasome assembly. To address this point, HMDMs were kept untreated or treated with CC, CC-C1q, or CC-C1q-vWF complexes for 18 h and the effect on caspase-1 activation was quantified with FLICA probes. Upon CC treatment, HMDMs showed a marked increase in FLICA signal, demonstrating caspase-1 activity (**Figure 5A**). While the presence of C1q on CC exhibited only a delicate reduction in caspase-1 activity, the additional presence of vWF significantly suppressed caspase-1 activity in HMDMs [median FLICA+ cells (IQR) in six independent donors of CC-C1q: 11.54% (7.29–28.38%) vs. CC-C1q-vWF: 9.37% (5.92–22.73%), $p = 0.0312$)] (**Figure 5B**).

Overall, our data show that HMDMs treated with CC-C1q-vWF complexes exhibit decreased caspase-1 activity that impacts on NLRP3 inflammasome dependent IL-1β secretion.

DISCUSSION

The cross-talk between the complement and the hemostatic systems is extensive and can provide synergistic benefits for the human body (38, 39). Yet, the role of many of these interplays is still unknown. In particular, even though an interplay between bound complement C1q and vWF has been demonstrated previously (17), its impact on the immune system has remained unexplored until now. In our study, we can illustrate that CC provide another physiological surface that allows a C1q-vWF interaction. Moreover, we found that the binding of vWF to bound C1q on CC is capable of modulating the immune response of macrophages by an upregulated expression of phagocytosis-mediating receptors and costimulatory receptors, a hampered phagocytosis and an enhanced suppression of pro-inflammatory cytokine secretion compared to C1q on CC alone.

Deposition of CC is described as a hallmark of atherosclerotic plaques. After recognition as DAMPs and ingestion by

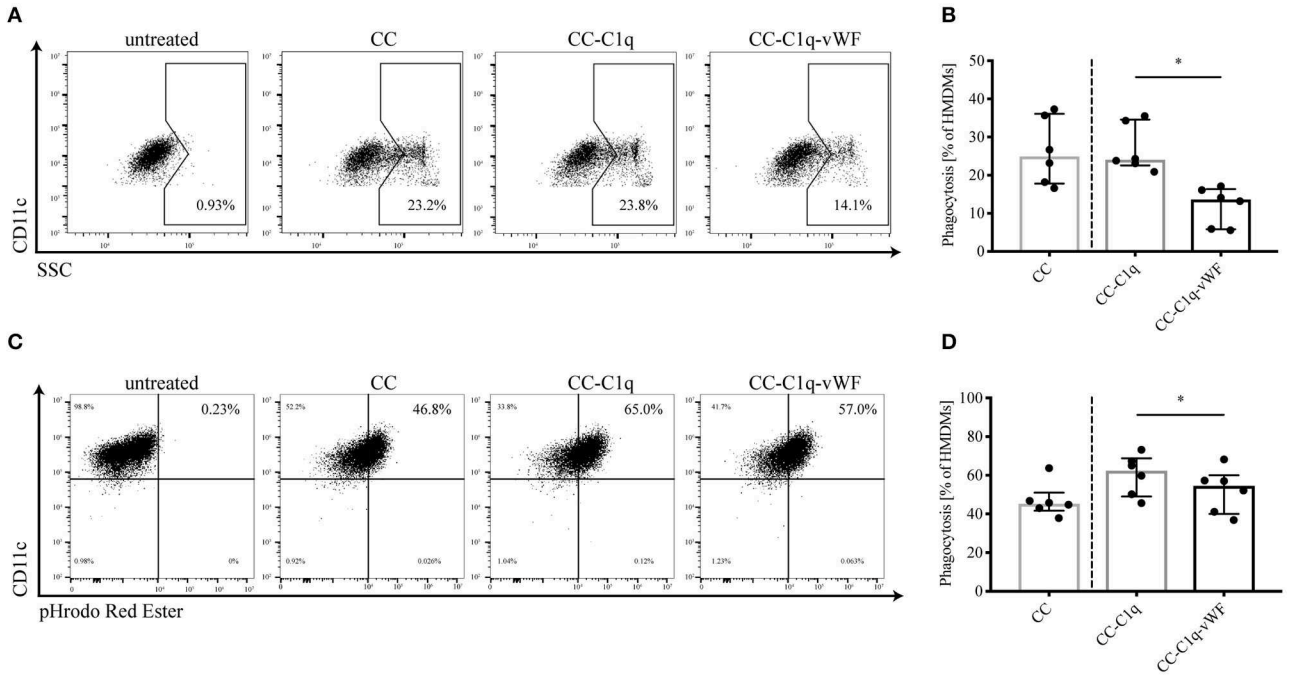

FIGURE 3 | Phagocytosis of CC-C1q-vWF complexes by HMDMs is hampered. **(A,B)** HMDMs were kept untreated or treated with CC, CC-C1q or CC-C1q-vWF complexes for 18 h and analyzed by flow cytometry for the degree of phagocytosis of CC complexes. Phagocytosis was determined by the percentage of CD11c+ cells with a shift into the SSC^{high} gate. **(A)** Flow cytometry dot plots show one out of six independent experiments. **(B)** Quantification of phagocytosis for pooled data is shown. **(C,D)** HMDMs were treated with CC, CC-C1q or CC-C1q-vWF pHrodo-dyed complexes for 30 min and analyzed by flow cytometry for the degree of phagocytosis of CC complexes. Phagocytosis was determined by the percentage of CD11c+ cells with a shift into pHrodo Red Ester+ gate. **(C)** Flow cytometry dot plots show one out of six independent experiments. **(D)** Quantification of phagocytosis for pooled data is shown. Columns denote median while error bars indicate interquartile range. Data points represent independent experiments analyzing six different healthy donors used to obtain HMDMs (Wilcoxon matched pairs signed rank test, $^*p < 0.05$).

phagocytes, CC trigger ROS formation and lysosomal leakage with consecutive NLRP3 inflammasome assembly, caspase-1 generation and IL-1β secretion (24). IL-1β secretion leads to further recruitment of phagocytes by an amplification loop in a concerted action with other pro-inflammatory cytokines and chemokines (40). Phagocytes, in particular macrophages, also are responsible for the essential function of recycling LDL and cholesterol in the periphery, but can develop into lipid-laden macrophage-derived foam cells during the course of the disease when their recycling capacity is overwhelmed. First, those foam cells can become apoptotic due to various stimuli, such as prolonged endoplasmic reticulum stress. Second, apoptotic cells that are insufficiently cleared (as occurring in advanced lesions due to defective efferocytosis), advance into cellular necrosis, in turn contributing to the formation of the necrotic core (41). Consequently, enhanced ingestion of LDL and CC fuels foam cell development, which is thought to be detrimental in later stages of atherosclerosis (42). Hence, the conclusion that CC induce arterial inflammation and destabilization of atherosclerotic plaques seems to be plausible (43). The complement molecule C1q can be considered as a double-edged sword in the context of atherosclerosis. Previous studies showed that the clearance of oxidized LDL and modified LDL is enhanced by binding of C1q (6), but simultaneously leads to a polarization of macrophages toward an anti-inflammatory phenotype through a reduction in pro-inflammatory cytokine secretion (44). In addition, C1q induces mRNA transcription of cholesterol efflux transporters (6). In contrast to these atheroprotective traits, C1q was demonstrated to be present on CC from human plasma (45) and found to be complexed to ApoE in human arteries (46), where it enables complement activation and thus contributes to atheroprogression (47, 48).

With regard to vWF, a number of studies in vWF-deficient animals and in patients suffering from Von Willebrand disease have been performed. Several of those animal studies (29) as well as human studies (49, 50) suggest atheroprogressive effects of vWF. Therefore, one could hypothesize disadvantageous consequences for the additional presence of vWF on CC-C1q complexes on macrophages. However, our findings unexpectedly point to a beneficial effect of vWF in the context of phagocytosis of CC by macrophages, and suggest that the role of vWF in atherosclerosis might be intricate and requires further investigation.

Previously, C1q and vWF have been regarded as separately acting molecules. Here, we identified not only a complex formation of C1q bound to the surface of CC but also the subsequent binding of vWF. Moreover, the treatment of HMDMs with CC-C1q-vWF complexes results in an upregulated

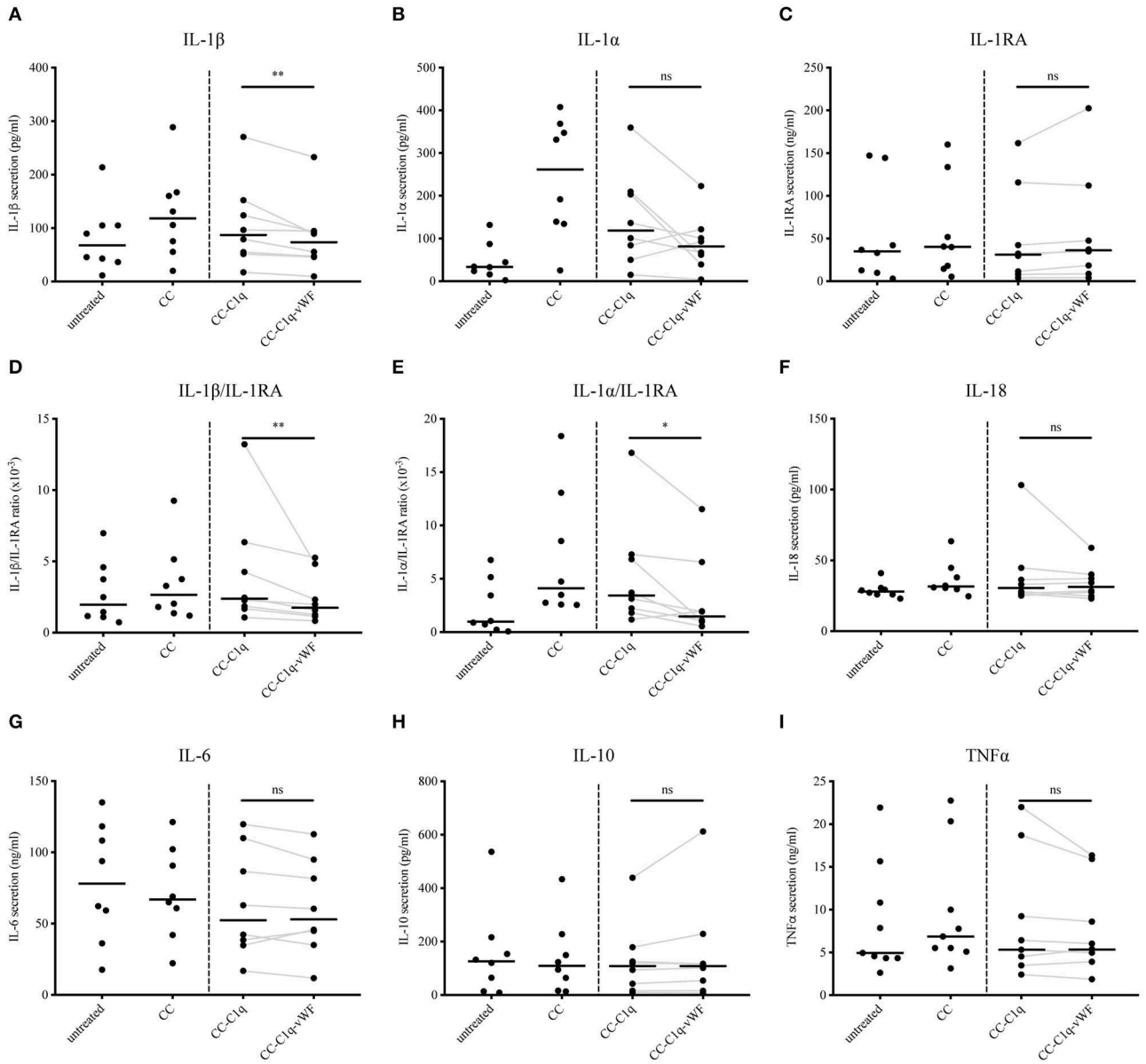

FIGURE 4 | CC-C1q-vWF complexes diminish LPS-induced IL-1 secretion of HMDMs. LPS-induced HMDMs were kept untreated or treated with CC, CC-C1q, or CC-C1q-vWF complexes for 18 h. Supernatants were analyzed by ELISA for cytokine secretion. Data show median cytokine concentrations of **(A)** IL-1β, **(B)** IL-1α, **(C)** IL-1RA, **(F)** IL-18, **(G)** IL-6, **(H)** IL-10, and **(I)** TNFα levels or median ratios of **(D)** IL-1β/IL-1RA and **(E)** IL-1α/IL-1RA of pooled donors. Data points represent independent experiments analyzing eight different healthy donors used to obtain HMDMs (Wilcoxon matched pairs signed rank test, $^{*}p < 0.05$; $^{**}p < 0.01$; ns, not significant).

expression of surface receptors of efferocytosis (MerTK), scavenger receptors (LRP-1 and SR-A1) as well as CD14, LAIR1, and PD-L1 compared to CC-C1q complexes alone. Studies investigating the role of the phagocytosis-mediating receptors MerTK and LRP-1 indicate atheroprotective features (51, 52), whereas the role of SR-A1 in cardiovascular disease is still controversial [reviewed by Ben et al. (53)]. Additionally, LAIR1 was described to have beneficial effects on foam cell formation (54). Therefore, we next sought to determine the effect on the phagocytic capacity of HMDMs. Interestingly, the presence of vWF on CC-C1q complexes strongly diminished the late as well as early phagocytosis of CC by HMDMs, hereby reversing the effect of C1q alone. A possible explanation for this unexpected finding could be that the upregulated expression of phagocytosis-mediating receptors is representing a reinforcing feedback loop that is triggered in order to compensate for the decreased ingestion of CC-C1q-vWF complexes. Last, our data illustrate a significant decrease in IL-1 cytokine secretion by HMDMs when

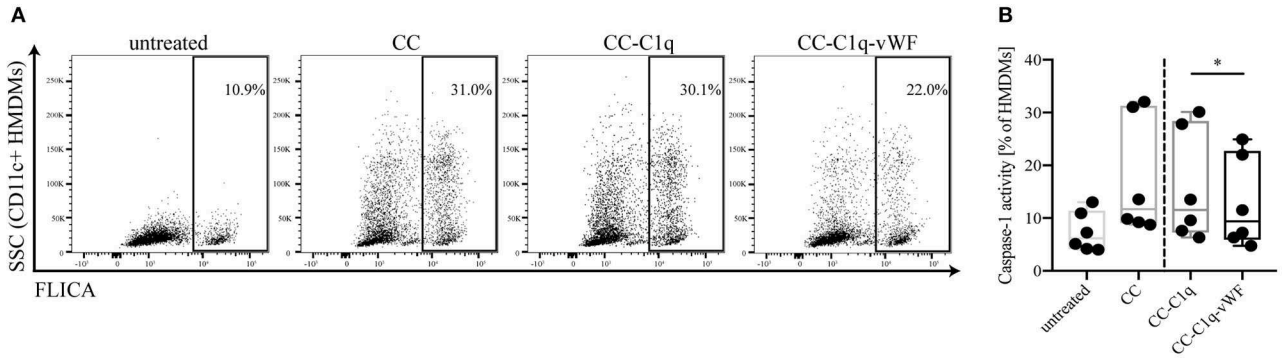

FIGURE 5 | CC-C1q-vWF complexes lead to a reduced caspase-1 activity in HMDMs. HMDMs were kept untreated or treated with CC, CC-C1q, or CC-C1q-vWF complexes for 18 h and analyzed for caspase-1 activity by flow cytometry. Activity of caspase-1 was determined by the percentage of CD11c+ cells in the FLICA+ gate. **(A)** Flow cytometry dot plots show one out of six independent experiments. **(B)** Quantification of caspase-1 activity for pooled data is shown. Horizontal lines in the box plots denote median while the boxes indicate interquartile range and whiskers minimum and maximum values. Data points represent independent experiments analyzing six different healthy donors used to obtain HMDMs (Wilcoxon matched pairs signed rank test, $*p < 0.05$).

treated with CC-C1q-vWF complexes compared to CC-C1q complexes without vWF. The clinical significance of IL-1 in cardiovascular disease was demonstrated by the anti-IL-1beta antibody Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) (55). Thus, a reduction in phagocytosis and inflammation could retard plaque progression (56, 57).

One limitation of our study is its *in vitro* character, since the *in vivo* situation in humans is likely to be more complex. C1q's role in human atherosclerosis is supported by studies that have shown C1q expression in atherosclerotic carotid arteries of patients (58–60) and therefore underlines the relevance of our results. Whereas the majority of C1q is non-covalently bound with serine proteases C1r and C1s to form the C1 complex in plasma and whole blood, free C1q is more prevalent in tissues where it is locally synthesized mainly by macrophages and dendritic cells (61). Furthermore, it has been demonstrated that vWF binds to a cryptic epitope of C1q, which is only exposed when C1q is surface-bound, while binding of vWF to surface-bound C1 was much weaker (17). Hence, we assume that the C1q-vWF interaction, especially on CC, primarily occurs in tissue, such as arteries of atherosclerotic patients. Nevertheless, further investigation on the occurrence of CC-C1q-vWF complexes in human atherosclerosis is needed.

Second, *in vivo,* shear stress is necessary to unfold the full functional potential of vWF (62). In our study however, permanent shear stress was not applied, since the physiological occurrence of shear stress would rather reflect the situation during plaque rupture resulting from continuous blood flow but not that inside the plaque itself.

Lastly, alternative ways can be envisaged by which the C1q-vWF interaction, in the form of CC-C1q-vWF complexes, might exert its effect on HMDMs. One of the ways could be partial steric shielding of the C1q molecule by vWF, weakening the effects of C1q (e.g., **Figure 3**). Another way could be an intrinsic effect of vWF (e.g., **Figures 2**, **4**). Future studies will have to explore the potential ways responsible for the overall impact of CC-C1q-vWF complexes. In addition, since the mutual interactions between complement and hemostatic systems *in vivo* are likely to be more complex, our *in vitro* model will have to be developed further in order to approach a physiological setting. Recently, Gravastrand et al. have described that CC induce complement-dependent activation of hemostasis (63). In our group, we have observed that complement activation remains unaffected by the presence of vWF (64). Hence, downstream complement components, such as C4 and C3, shall be implemented into our system and its effect on HMDMs in the additional presence of platelets addressed in the future.

In conclusion, with this study, we provide new insights into an emerging cross-talk between C1q and hemostasis-initiating vWF. Our findings reveal that binding of vWF to C1q on CC regulates the immune response of HMDMs. We show that CC-C1q-vWF complexes provoke a hampered phagocytosis together with an accompanied reduction of IL-1 cytokine secretion by macrophages that could prove favorable for retarding foam cell formation and decelerating plaque progression.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

CD designed and performed the study, collected and analyzed data, and wrote the manuscript. ST and MT designed and supervised the study, and critically revised the manuscript.

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REVIEW
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The Complement System and C1q in Chronic Hepatitis C Virus Infection and Mixed Cryoglobulinemia

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The complement system bridges innate and adaptive immunity against microbial infections, with viral infection being a major trigger. Activation of the classical, alternative, and lectin pathways have been reported in chronic hepatitis C virus (HCV) infection and/or cryoglobulinemia. HCV infection leads to dysregulation of complement-mediated immune responses. Clinical and experimental evidence support involvement of complement in intra- and extrahepatic manifestations of HCV infection, such as liver fibrosis and type II cryoglobulinemia. In this review, we summarize studies that have investigated the interplay between HCV and the complement system to establish chronic infection and autoimmunity, as well as the association between HCV pathogenesis and abnormal complement profiles. Several unanswered questions are highlighted which suggest additional informative lines of investigation.

Keywords: liver, hepatitis C virus, complement, C1q, gC1qR, mixed cryoglobulinemia

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INTRODUCTION

The complement system includes major host defense mechanisms that bridge innate and adaptive immunity against microbial infections. It is also a critical mediator of the clearance of immune complexes and injured cells (1–3). Dysregulation may be associated with chronic autoimmune inflammatory conditions, such as systemic lupus erythematosus, cryoglobulinemia, and rheumatoid arthritis. Viral infections may trigger dysregulation by direct effects on complement components for the purpose of immune evasion, by effects on specific receptors used for viral entrance into cells or by promoting a pathogenic antiglobulin [i.e., rheumatoid factor (RF)] response as part of chronic immune stimulation. In particular, hepatitis C virus (HCV) infection has been associated with a number of extrahepatic disorders, such as type II mixed cryoglobulinemia (MC) and B cell lymphoma that may be accompanied by complement dysregulation. The recent introduction of direct-acting antiviral (DAA) therapy for HCV allows most patients to achieve a sustained virological response (SVR)/cure. Treatment reduces liver inflammation and improves extrahepatic disease manifestations, but cryoglobulinemia and liver dysfunction may persist (4–14).

The majority of HCV-infected patients with evidence of B cell clonality have abnormal complement profiles; a low serum level of C4 is a "signature" for type II MC patients (15, 16). However, only limited information is available regarding the mechanisms by which the complement system is involved in HCV-induced intra- and extrahepatic disease. Therefore, in this review, we aim to highlight the interplay between HCV and the complement system that become apparent with chronic infection and lymphoproliferation.

PRODUCTION SITES OF COMPLEMENT PROTEINS AND RECEPTORS

More than 40 complement-related proteins have been identified in the plasma and on cell surfaces, constituting more than 15% of the of plasma globulins (17, 18). The liver makes ~90% of the plasma components of classical, alternative, and lectin pathways. By contrast, whereas hepatocytes are the predominant source of C1r and C1s in blood, activated monocytes/macrophages and immature dendritic cells are the primary source of C1q, a recognition molecule for classical pathway activation that also has significant non-complement functions (19). Although the liver produces the majority of C4, multiple tissues may also produce this protein for local consumption, particularly in response to interferon-gamma (20). Several complement receptors, such as complement receptor type 1 (CR1), the complement receptor of the immunoglobulin superfamily (CRIg), the integrin complement receptors 3 (CR3) and 4 (CR4), and the complement component 5a receptor 1 (C5aR), are expressed on liver cells (hepatocytes, endothelial cells, Kupffer cells, and stellate cells), and contribute to a variety of functions, such as induction of gluconeogenesis, synthesis of acute-phase proteins, hepatocyte proliferation, and phagocytosis (21).

FIBROSIS AND REGENERATION

Increasing evidence implicates activation of the complement system in the pathogenesis and response to acute and chronic liver injury (22). In a clinical study investigating the relationship between complement activation and development of liver fibrosis, blood samples from 50 chronically infected HCV patients were compared to 35 patients with other various liver diseases and 50 healthy controls (23). Complement system activation, as indicated by a significant decrease in total plasma complement activity (CH50) and increase in SC5b-9 [a marker for generation of the membrane-attack complex (MAC)] was associated with high necroinflammatory activity in the HCV patients and patients with other liver diseases compared to controls. The level of SC5b-9 significantly correlated with liver fibrosis stage in HCV-infected patients but not in patients with other liver injuries. In a proteomic survey of serum samples from HCV-infected patients, liver fibrosis stage was associated with a decrease in C3, C4, and Factor H-related protein-1, a regulatory C3b-binding protein (24). C4a, a cleavage product of C4 that contrasts with C3a and C5a with regard to biologic function, was reported to be negatively correlated with the stage of liver fibrosis in children with chronic HCV infection, serum levels being significantly lower in HCV children with advanced fibrosis than those with no/mild fibrosis (25); the significance of this study, however, was limited by the low numbers of HCV-infected children, and the fact that C4a may increase as a function of classical pathway activation and C4 consumption. In a murine genomic study, the gene encoding C5 was identified as a quantitative trait locus associated with the development of liver fibrosis (26). Expression of C5aR1 was significantly upregulated on hepatic stellate cells during transdifferentiation to myofibroblasts in culture. Since myofibroblasts synthesize collagens and other extracellular matrix proteins, elevated C5aR1 expression is consistent with the concept that C5 is a modifier of liver fibrogenesis. Indeed, blockade of C5aR1 reduces hepatic fibrosis in mice (26). Critical roles for C3a and C5a for liver regeneration following carbon tetrachloride injury have been shown in C3, C5, and C3aR knock-out and double knock-out mice, reversible by restoration of C3a, C5, or C5a (27). Interestingly, in 277 patients with chronic HCV infection, two C5 single-nucleotide polymorphisms (SNPs), rs17611 and rs2300929, were associated with advanced fibrosis (26). However, in a study of 1,435 HCV-infected patients and 1,003 patients with other liver diseases, there was no significant association between these C5 SNPs and fibrosis in either patient group (28).

HEPATOCELLULAR CARCINOMA (HCC)

Cancer growth is determined by intrinsic properties of malignant cells as well as several modifiers, including the complement system, which may either reduce or increase progression (29). HCC is the second leading cause of organ-specific cancer-related death worldwide (30) and is the most rapidly increasing cause of cancer-related death in the United States, Europe, and Japan (31). Of note, C3a was identified by mass spectrometry and 2-dimensional gel electrophoresis (2-DE) of serum samples obtained from HCV-HCC, chronic HCV, HBV-HCC, chronic HBV, and healthy subjects as a differentially expressed biomarker protein with significantly higher levels among HCV-HCC patients compared to the other groups (32).

TREATMENT

Until recently, HCV treatment has centered on interferon-alpha (IFNα), a cytokine with both antiviral and immunologic effects. However, IFNα-based treatment failed to eliminate HCV in many patients and was often poorly tolerated, particularly because of its ability to induce, uncover and/or exacerbate autoimmune/inflammatory disorders (33, 34). The CC genotype associated with the rs285009 SNP of the C4 gene closely correlated with decreased level of mRNA expression and C4 protein which was more striking at baseline in HCV patients compared to healthy controls. More importantly, the presence of this SNP was significantly associated with a poor response to IFNα-based therapy as well as the development of a high degree of liver fibrosis (35). Interestingly, a significant reduction in C4 activity was also observed in relapsers after IFNα treatment compared to patients who achieved SVR (36). Polymorphism at the rs2230201 SNP of C3 was also associated with IFNα treatment outcome (37). The rs2230201 'C' allele was associated with increased serum C3 levels compared to the 'T' allele, which conferred an advantage in attaining SVR, especially in homozygotes. Patients with serum C3 value < 53 mg/dL and non-CC genotypes may not respond to IFNα treatment (37). Recently developed DAA therapies provide an opportunity for HCV patients with autoimmune/inflammatory disorders to be cured with a low risk of side effects (38–40). In the era of DAA, how a patient's complement profile contributes to the treatment response remains to be defined.

In summary, accumulating clinical observations support a role for the complement system in mediating liver inflammation and fibrosis in HCV infection (**Table 1**). However, the mechanisms

TABLE 1 | Complement system abnormalities in HCV-induced liver injury.

Complement abnormality	HCV-induced liver injury	Reference
Low plasma CH50	High necroinflammatory activity	(23)
High SC5b-9	High stage of necroinflammation and degree of liver fibrosis	(23)
Low C3	High degree of liver fibrosis	(24)
Low C4	High degree of liver fibrosis	(24, 25)
CC genotype of rs285009 single nucleotide polymorphism (SNP) of the C4 gene	High degree of liver fibrosis and poor response to IFNα-based therapy	(35)
Low factor H-related protein-1	High degree of liver fibrosis	(24)
High C5	High degree of liver fibrosis	(26)
High C3a	High risk of hepatocellular carcinoma	(32)
Low serum C3 and non-CC genotype of rs2230201 SNP of the C3 gene	Poor response to IFNα-based therapy	(37)

TABLE 2 | HCV strategies to evade innate and adaptive immunity using complement system-related components.

Complement system-related factor	HCV-induced immune evasion	Reference
C3	Downregulation of C3 promotor activity by HCV-NS5A *via* inhibition of C/EBP-β	(43)
C2	Impairment of C3 convertase function *via* inhibition of C2	(44)
C4	Inhibition of C4 activity through HCV core-induced inhibition of upstream stimulatory factor-1 and HCV-NS5A-induced inhibition of interferon regulatory factor-1	(45)
C9	Impairment of membrane-attack complex (MAC) formation through inhibition of C9 promotor activity by HCV-core	(46)
C3 and C4	Downregulation of C3 and C4 hepatocyte synthesis through the inhibition of the hepatocyte MICA/B	(47)
MAC	Impairment of MAC formation through incorporation of CD59 in HCV envelope	(55)
C3 convertase	Upregulation of CD55 expression which accelerates the decay of C3 convertase	(56)
gC1qR	Impairment of T-cell immunity through the interaction of HCV core to gC1qR on T-cells and monocyte-derived dendritic cells	(65–67)

underlying these observations are still unclear. Thus, further experimental and molecular studies are required to dissect how the complement system contributes to intrahepatic HCV pathogenesis, including roles in innate and adaptive immunity, regulation of apoptosis, fibrosis, and regeneration.

HCV STRATEGIES TO OVERCOME ANTIVIRAL RESPONSES OF THE COMPLEMENT SYSTEM

Hepatitis C virus lacks a DNA intermediate; thus, it is incapable of integrating into host chromosomal DNA. Despite this, unlike most RNA viruses, chronic infection is established in ~80% of cases (41) through multiple strategies to evade innate and adaptive antiviral responses (42). In part, this is accomplished directly by inhibition of complement components and/or indirectly by induction of regulators of complement activation (RCA) (**Table 2**). Mazumdar et al. examined the relationship between HCV infection and C3 concentrations in blood. C3 has a central role in modulating all three pathways of the complement system. In matched serum and liver biopsy samples from HCV patients, both the levels of C3 in serum and the expression of mRNA in biopsies were significantly lower compared to serum and tissue obtained from healthy donors (43). Further *in vitro* studies showed that HCV-NS5A protein strongly downregulated C3 promoter activity at the basal level. In addition, expression of the transcription factor C/EBP-β, which induces C3 promoter activity, was reduced in immortalized human hepatocytes and human hepatoma cells (Huh7) that were either infected with cell culture-adapted HCV or transfected with HCV-NS5A (43). Moreover, HCV inhibited C3 convertase activity, which is critical in promoting the activity of classical and lectin pathways of complement system (44). Infection of a hepatoma cell line with HCV resulted in inhibition of C2 expression and hence impairment of C3 convertase function. On the other hand, C3b deposition onto bacterial membrane was reduced by sera from HCV patients as compared to healthy controls, which further indicates impaired C3 convertase (44). C4 contributes to the eradication of several viral infections by its role as opsonin and by its central role in promoting the activity of the classical and lectin pathways (3). Notably, C4 protein levels in the serum and mRNA expression levels in liver tissue were lower in HCV patients compared to patients with unrelated liver diseases (45). *In vitro* studies showed that the expression levels of the two C4 isoforms (C4A and C4B) were significantly reduced in hepatocytes transfected with a full-length HCV genome. In particular, among different HCV proteins, only core and NS5A contributed to HCV's inhibitory effect on C4 as shown by *in vitro* transfection experiments, using the Huh7 hepatoma cell line and plasmids containing different HCV proteins (45). Consistent with these *in vitro* results, the expression levels of C4 mRNA in liver tissue of HCV-core or NS5A transgenic mice were also significantly reduced. Mechanistic studies showed that HCV-core downregulated the expression of upstream stimulatory factor-1, a transcription factor critical for C4 expression, while NS5A inhibited the expression of interferon regulatory factor-1, which is required for IFN-γ-induced C4 promoter activation (45).

Likewise, Kim et al., showed that liver biopsies from HCV patients had lower expression of C9 mRNA compared to samples from unrelated diseases or healthy controls. This indicates that HCV regulates the MAC *via* C9. C9 mRNA was significantly downregulated in cultured hepatocytes infected with HCV (46). In particular, HCV-core protein had a critical role in regulating C9 promoter activity. Furthermore, in a subsequent *in vitro* study, HCV NS2 and NS5B proteins were found to be responsible for

HCV-associated inhibition of the hepatocyte protein major histocompatibility complex class I-related chains A and B (MICA/B) which functions as a key receptor ligand for NKG2D on NK cells resulting in downregulation of C3 and C4 hepatocyte synthesis (47).

A general role for lectins and pattern recognition of viral glycoproteins has been identified for HIV and HCV (48). *In vitro* studies showed that mannan-binding lectin (MBL) bound to the HCV E2 ectodomain and E1/E2 heterodimers through its lectin domain, as well as activate complement through MBL-associated serine protease 2 (49). Ficolin-2, a known lectin pathway activator was found to inhibit attachment of HCV envelope E1 and E2 N-glycans to their low-density lipoprotein (LDL) and scavenger B1 receptors (50), with elevated blood levels of L-ficolin or MBL being found in the serum of some patients, possibly correlating with MBL2 genetic variants and response to IFNα (51).

Host expression of RCAs, such as CD35, CD46, CD55, and CD59, serves to protect the cells from MAC lysis (52, 53). HCV has developed strategies to attenuate complement activation by regulating RCAs. Amet et al. first showed that CD59, a key member of RCA, associated with the external membrane of HCV particles obtained from infected patients and Huh7.5.1 cells and had a direct role in abrogating antibody-dependent complement-mediated lysis (54). *In vitro* studies by Ejaz et al. indicated that HCV selectively incorporates CD59 in its envelope, which inhibits the formation of the MAC complex (55). Also, it was found that HCV infection upregulates the expression of CD55, which accelerates the decay of C3 convertase (56). Taken together, HCV has the capability to attenuate the complement system at multiple steps to weaken the innate immune response.

ROLE OF gC1q RECEPTOR (gC1qR) IN HCV PATHOGENESIS

gC1q receptor is an acidic multifunctional cellular protein ubiquitously expressed on somatic cells (57). It binds to the globular heads of C1q and modulates complement activation (58). Apart from its interaction with C1q, gC1qR binds to several host cell-surface ligands, such as vitronectin and high molecular weight kininogen (59). Interaction of these ligands with gC1qR leads to classical complement pathway activation with generation of inflammatory cytokines, cell adhesion, and activation of the intrinsic coagulation cascade leading to the production of bradykinin, increased vascular permeability, and infiltration of vascular tissue with proinflammatory cells (60). In addition to cellular proteins, gC1qR interacts with several microbial proteins, such as adenovirus core protein (61), HIV rev (62), and protein A of *Staphylococcus aureus* (63), suggesting its role in the pathogenesis of these infections. Interestingly, by using HCV-core protein as bait in yeast two-hybrid assay, Kittlesen et al. was the first to report the interaction between gC1qR and HCV-core (64). The interaction of HCV-core to gC1qR on T-lymphocytes resulted in inhibition of T-cell proliferation and function through impairment of ERK/MEK phosphorylation (65) and Lck/Akt activation (66). Also, engagement of gC1qR on monocyte-derived dendritic cells with HCV-core resulted in an impaired capacity to generate type 1 $CD4^+$ T cell immunity *via* inhibition of TLR-induced IL-12 production (67). Therefore, HCV might utilize the direct interaction of its core protein with gC1qR on T cells as a tool to suppress cellular immunity which might imply an important role in persistent infection, an observation that might extend to minicore isoforms of this protein, which lack the RNA binding domain of the p21 core (68).

By contrast to the inhibitory influence of HCV-core protein and gC1qR interaction on T cell responses, this interaction on B cells resulted in hyper-activation and proliferation indicated by upregulation of CD69, overexpression of costimulatory and chemokine receptors, and increased production of IgM and IgG (69). This might partially explain the link between chronic HCV infection, B-cell lymphoproliferative disorders, and several autoimmune-related diseases (70–72). In support of this, the level of circulating gC1qR and gC1qR mRNA of PBMC in HCV patients with MC, one of the major B-cell disorders associated HCV infection, is significantly increased compared to HCV patients without MC or healthy controls (73). Interestingly, there was also a positive correlation between circulating gC1qR with RF activity and C1q concentrations in HCV patients with MC (73). Taken together, these observations suggest the involvement of gC1qR in the pathogenesis of HCV-induced autoimmunity.

ROLE OF C1q IN HCV-INDUCED MC

Mixed cryoglobulins are cold-precipitable complexes of monoclonal or polyclonal IgM RF with polyclonal or oligoclonal IgG (15). Type II MCs, which are composed of monoclonal IgMκ RF and polyclonal IgG, are heavily represented among cryoglobulins associated with chronic HCV infection, and those found in patients with primary Sjogrens syndrome, both of which may be complicated by clonal B-cell proliferations and specific types of non-Hodgkin's lymphoma (15). HCV patients with symptomatic type II MC suffer from various extrahepatic manifestations, including vascular, renal, and neurological lesions (74), i.e., cryoglobulinemic vasculitis (CryoVas) (75).

In type III MCs, both the IgM and IgG components appear to be polyclonal; extrahepatic disease manifestations may occur, but cryoglobulin levels are lower than type II, and an association with asymptomatic disease is more frequent. In addition, intermediate types characterized by oligoclonality or mixed IgM clonality with polyclonal IgM (type IIa) have also been described (76). Type III MCs may be found in HCV infection, as well as associated with rheumatic diseases [e.g., systemic lupus erythematosus (SLE)] in which complement activation may occur (16). The significance of Type IIa and related intermediate forms with regard to the progression to clonality that may occur in cirrhosis associated with HCV, and in primary Sjogrens syndrome, remains to be fully defined (77, 78).

As noted, a low serum level of C4 is a significant "signature" of type II MC patients (15, 16). This selective depression of C4 strongly implicates classical pathway activation of the complement in cryoglobulin formation. However, the level and function of C4 may be significantly influenced by inter-individual copy-number variation of C4A or C4B genes, charge variation, or isotype deficiency of these genes (79). Incorporation of C1q into isolated cryoprecipitates was first demonstrated as an 11S peak on density gradient ultracentrifugation in patients with lupus nephritis (80).

More recent studies have confirmed that cryoprecipitates from patients with HCV-associated CryoVas are enriched in C1q (81), antibodies to HCV antigens (82), and may contain HCV-core protein as indicated by results obtained using an enhanced highly sensitive chemiluminescent microparticle immunoassay (81). Based on evidence that C1q and HCV-core bind to gC1qR, gC1qR/HCV-core complexes might provide a platform for complement activation and deposition of C4D at sites of vasculopathy (83). Additional factors that might be reflected in depletion of C4/C1q and localization to cryocomplexes include the ability of C1q to bind promiscuously to >100 known ligands, including both IgG- and IgM-containing immune complexes, surface-bound C-reactive protein, and molecules exposed at the surface of apoptotic cells, with binding through charged residues on the apex of the gC1q heterotrimer (19, 84), acquired C1-inhibitor deficiency (85), regulation of activation by C4-binding protein (86, 87), and antibodies to C1q and/or potentially to other components of the C1 complex (88).

ROLE OF RF IN MC PATHOGENESIS

Although MC may be associated with a rheumatoid-like arthritis, it is distinct from RA in that antiglobulin activity is restricted to the IgM isotype; although it is presumed that the antigen specificity is directed primarily to determinants in the Fc portion of IgG uncovered by aggregation or complexing to antigen, there have been some suggestions of F(ab)′2 anti-hinge or anti-idiotypic specificity. Low-affinity RFs (Kd~10^{-5}M) are natural antibodies cross reactive with other autoantigens, whereas high-affinity RFs (Kd~10^{-7}M) have undergone affinity maturation and are hypermutated (89). The RF response is broadly represented in a number of infectious etiologies, either as a response to immune complexes formed by the infecting microbe and antibodies or as a function of direct infection and polyclonal B-cell stimulation. In Type II cryoglobulins, the contribution of IgM and IgM RF to total protein content presents a spectrum of concentrations and clonality, ranging from cryocomplexes with RF activity greatly exceeding serum levels and enhanced representation of IgM cross-reactive idiotypes (notably the Wa idiotype first described 45 years ago) (90). This allows for the observation of RF in mixed cryoglobulins in patients with apparently negative RFs, and mixed cryoglobulins that are composed almost entirely of IgM with kappa light chains. It has also been reported that HCV virion in fractionated mixed cryoglobulins is complexed with VLDL, providing a mechanism for localization of virus to sites of pathology *via* the LDL receptor (91), and the potential for LDL receptor genetic polymorphisms to influence disease outcome (92). RFs studied from B-cell clusters isolated by microdissection of liver biopsies from HCV-infected patients were hypermutated and overlapped in regard to variable region gene usage with blood and bone marrow (93).

APOPTOTIC ROLE OF C1q IN SLE

Activation of complement is a central feature of SLE, intimately related to the pathogenesis of lupus nephritis, and a marker for disease activity and relapse; deficiencies or polymorphisms of molecules central to the classical, alternative, and lectin pathways have been linked to disease susceptibility, immune-complex nephritis, and severity (94). In particular, a central role for C1q and C1q receptors, both with regard to deficiency and as molecular cell-surface sensors for innate and acquired immune responses, has been reviewed (95). The observation that SLE develops in ~90% patients genetically deficient in C1q highlighted a function of C1q as "protector" against autoimmunity that may be independent of its classical role ion complement activation (96). In SLE, C1q deficiency may result either from genetic disorders or anti-C1q autoantibodies (97). The contributions of C1q deficiency in the development of SLE can be related to abrogation of binding to molecules (phosphatidylserine, double-stranded DNA, glyceraldehyde-3-posphate dehydrogenase, annexins 2 and 5, calreticulin) expressed on the surface of dying cells (17) and resultant lack of generation of activated C1s to cleave these apoptotic autoantigens (19). In addition, all three pathways of complement can be activated on the surface of apoptotic cells without further activation of innate or adaptive immune components (17); impaired clearance of dying cells and immune complexes in absolute or functional C1q deficiency is linked to the development of self-reactive B cells with affinity toward multiple autoantigens, and effects on monocyte and dendritic cell differentiation (98, 99).

Anti-C1q autoantibodies have been reported in 30–50% of SLE patients, most commonly correlating with antibodies to double-stranded DNA, nephritis, and low levels of C3 and C4 (100, 101). While antibodies with unique specificities for the globular head and collagen tail of C1q have been identified, the impact of blocking C1q domains on biological activity remains uncertain compared to a number of other (e.g., calreticulin) known inhibitors (17). Although the C1q is a major component of HCV-induced cryoprecipitate, to the best of our knowledge there are no published studies addressing this issue. Low levels of C1q and a significant prevalence of anti-C1q autoantibodies are shared features of SLE and HCV-induced cryoglobulinemia (36, 73, 102, 103).

Interferon-alpha is well-known to have both antiviral and inflammatory effects (104). Plasmacytoid dendritic cells (pCD) are the major producers of IFNα (105). Interestingly, C1q collagen tail interacts with LAIR-1 (CD305), an inhibitory receptor for C1q (106), on pCDs and restricts the production of IFNα (107). Therefore, anti-C1q autoantibodies might contribute to HCV-induced cryoglobulinemia by blocking the interaction between the C1q tail and its inhibitory receptor, LAIR-1, on pDCs resulting in uncontrolled overproduction of IFNα, which may in turn drive the inflammation associated with progression of MC in HCV patients. Alternatively, elevated levels of IFNα produced by uncontrolled pCDs might promote differentiation of B cells into plasma cells resulting in production of pathogenic autoantibodies reported in SLE (108).

CONCLUSION

The complement system plays a central role in rheumatic and autoimmune diseases, several of which are associated with the presence in blood of cold-perceptible immune complexes enriched in IgM RF, specific antibody activities, putative antigens and C1q as part of a cascade capable of activating the classical pathway, leading

in turn to the generation of anaphylatoxins, chemotactic factors, and inflammatory mediators. Both C1q and its globular receptor are promiscuous with regard to ligand specificity, allowing for alternative functions that include binding to specific intracellular antigens expressed on the surface of apoptotic cells, as well as to specific domains of HCV. A research agenda includes the mapping of C1q epitopes responsible for binding to diverse ligands, anti-C1q antibodies, heterotrimeric formation, and C4/C2 serine protease generation that might in turn be targets for therapy. Similar therapeutic strategies might be targeted to gC1qR binding to C1q and High Molecular Weight Kininogen in plasma, on the surface of endothelial cells as a mechanism for vasculopathy, and the regulation of danger sensors on mononuclear cells and immature dendritic cells. A second line of investigation is the delineation of factors responsible for the strikingly low C4 levels in sera of patients with Type II MC and some patients with SLE with regard to mechanisms such as copy-number variation, polymorphisms, cleavage and deposition in tissue, and specific inhibitors. With regard to HCV, a focus on liver pathology would provide an arena to identify complement-defined mechanisms of disease, including immune activation in lymphoid follicles, steatosis, fibrosis, and regeneration.

AUTHOR CONTRIBUTIONS

AE and PG wrote the review. AB and TS revised the manuscript.

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Complement Protein C1q Binds to Hyaluronic Acid in the Malignant Pleural Mesothelioma Microenvironment and Promotes Tumor Growth

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C1q is the first recognition subcomponent of the complement classical pathway, which acts toward the clearance of pathogens and apoptotic cells. C1q is also known to modulate a range of functions of immune and non-immune cells, and has been shown to be involved in placental development and sensorial synaptic pruning. We have recently shown that C1q can promote tumor by encouraging their adhesion, migration, and proliferation in addition to angiogenesis and metastasis. In this study, we have examined the role of human C1q in the microenvironment of malignant pleural mesothelioma (MPM), a rare form of cancer commonly associated with exposure to asbestos. We found that C1q was highly expressed in all MPM histotypes, particularly in epithelioid rather than in sarcomatoid histotype. C1q avidly bound high and low molecular weight hyaluronic acid (HA) *via* its globular domain. C1q bound to HA was able to induce adhesion and proliferation of mesothelioma cells (MES) *via* enhancement of ERK1/2, SAPK/JNK, and p38 phosphorylation; however, it did not activate the complement cascade. Consistent with the modular organization of the globular domain, we demonstrated that C1q may bind to HA through ghA module, whereas it may interact with human MES through the ghC. In conclusion, C1q highly expressed in MPM binds to HA and enhances the tumor growth promoting cell adhesion and proliferation. These data can help develop novel diagnostic markers and molecular targets for MPM.

Keywords: complement system, malignant pleural mesothelioma, hyaluronic acid, mesothelioma cells, C1q, cancer

Abbreviations: MPM, malignant pleural mesothelioma; MES, mesothelioma cells; ECM, extracellular matrix; HA, hyaluronic acid; ghA, ghB and ghC: globular head region of human C1q A, B and C chain, respectively; MBP, maltose-binding protein; HMW, high molecular weight; LMW, low molecular weight.

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a rare form of cancer that develops from cells of the pleural mesothelium and is most commonly associated with exposure to asbestos (1). MPM typically develops after a long latency period, which averages 30–40 years, and the average age of patients is 60 years (2). MPM is highly invasive to surrounding tissues leading to the failure of the organs underlying the serosal membranes (3). Although low in metastatic potential, metastasis in MPM are more frequent postsurgery; at the autopsy, metastatic diffusion is observed in 50% of patients (3). Microscopically, MPM shows mainly one of the three patterns: epithelioid, sarcomatoid, or biphasic (4).

MPM is an aggressive malignancy; most patients succumb within 2 years of being diagnosed (5). Treating MPM remains a challenge. There are two main treatment alternatives: palliative chemotherapy or multimodal treatment including surgical resection combined with chemotherapy or radiotherapy, or both (6). The resistance of MPM to conventional treatment and poor prognosis has renewed interest in basic research in order to understand the MPM biology fully with the aim of identifying possible new molecular therapeutic targets.

The local microenvironment, which encourages survival, growth, and invasion of cancer cells, plays a critical role in cancer development; the extracellular matrix (ECM) is an essential constituent of such microenvironment (7). Hyaluronic acid (HA), a member of the glycosaminoglycan family, is an abundant and ubiquitous component of the ECM (8). HA is a negatively charged high-molecular-weight (HMW) polysaccharide (4–800 kDa) which is made up of the repeating disaccharide (glucuronic acid and *N*-acetylglucosamine) (9). In the tumor microenvironment, HA offers a molecular 3D-scaffold for cells *via* the assembly of ECM, thus modulating stromal as well as tumor cells (10). HA, whose multiple functions are dictated by its molecular size and tissue concentration, relies on balanced biosynthetic and degradation processes. Increased HA synthesis has been associated with cancer progression and metastasis (11). In patients with MPM, large quantities of HA are found in the tumor tissue although both malignant and benign mesothelial cells have been found positive for intracytoplasmic HA (12).

The complement system also constitutes the local environment for cancer as an immune surveillance against malignant cells due to its ability to promote inflammation and causes direct cell killing (13). We focused our investigation on C1q, which is the first recognition subcomponent of the complement classical pathway. C1q is a potent link between innate and adaptive immunity by virtue of its ability to bind IgG- and IgM-containing immune complexes (14). In addition to being involved in the clearance of apoptotic cells, and thus maintenance of immune tolerance, C1q also has the ability to directly impact upon cell differentiation and proliferation, dendritic cell maturation, and synaptic pruning; functions that are not reliant on complement activation by C1q (15). Recently, involvement of C1q in pregnancy *via* its ability to modulate the endovascular (16) and interstitial invasion (17) of trophoblast cells in placenta has also been demonstrated. In addition, we have recently showed that C1q is present in several solid human tumor tissues and is involved in tumor progression (18).

The present study focused on the involvement of C1q in the proliferation and invasiveness of MPM. We found that C1q can bind to HA and acquires protumorigenic properties, leading to heightened adhesion, migration and proliferation of human mesothelioma cells (MES).

MATERIALS AND METHODS

Reagent and Antibodies

Hyaluronic acid was a kind gift from Prof. Ivan Donati, Department of Life Sciences, University of Trieste (19). C1q was either purified from fresh human serum following the procedure as described previously (20) or bought from Sigma-Aldrich (Milan, Italy). The recombinant globular head regions of the A, B, and C chains (ghA, ghB, and ghC, respectively) were expressed as fusion proteins linked to maltose-binding protein (MBP) in *Escherichia coli* BL21 and purified, as described previously (21). Poly-L-lysine, bovine serum albumin (BSA) and all reagents were from Sigma-Aldrich. The following antibodies were used: monoclonal antibody (mAb) mouse anti-human C1q was from Quidel (Quidel Corporation, San Diego, CA, USA), sheep anti-human C1q and anti-human C4 were purchased from The Binding Site (Bergamo, Italy). Mouse Monoclonal anti-C5b-9 antibody (aE11) was from AbCam. Mouse mAb anti-human von Willebrand factor (vWF), mouse mAb anti-human CD68, rabbit anti-human C1q, and goat anti-mouse-FITC F(ab)′ were purchased from Dako (Milan, Italy). Mouse mAb anti-human CD44-PE, mouse mAb anti-human CD45-PE-, or FITC-conjugated, unrelated mouse IgG1-PE- and FITC-conjugated were from Immunotools (Friesoythe, Germany). Cy3-conjugated $F(ab')_2$ goat anti-mouse IgG, and FITC-conjugated $F(ab')_2$ goat anti-rabbit IgG. Mouse mAb anti-human Mesothelin and rabbit anti-human Calretinin were from Santa Cruz Biotechnology (DBA, Milan, Italy). Mouse monoclonal anti-human Vimentin, goat anti-mouse IgG alkaline phosphatase (AP)-conjugated, anti-rabbit IgG-AP-conjugated, and anti-goat IgG-AP-conjugated were from Sigma-Aldrich.

Patients and Specimens

MPM patients who were diagnosed and followed up at the Department of Pneumology, University Hospital of Cattinara, Trieste, Italy, were enrolled for this study. None of the patients received chemotherapy or radiotherapy prior to sampling.

Patients (five male) with reported asbestos exposure underwent pleuroscopy for diagnosis of pleural effusion. All the procedures were performed under conscious sedation achieved by titration of intravenous midazolam and meperidine. Before the procedure, patients were placed in the lateral decubitus position with the pleural effusion uppermost and a bedside chest ultrasonography was used to determine the entry site. After the creation of the sterile field and injection of 2% lidocaine in the intercostal space in order to obtain local anesthesia, a 2-cm skin incision was made with a scalpel, then blunt dissection of the chest wall was performed using curved Kelly forceps down to the parietal pleural. Finally, a trocar was placed into the pleural

space and the pleuroscope (Karl Storz GmbH, Tuttlingen, Germany) was inserted to examine parietal and visceral pleura to obtain parietal pleural specimens using dedicated forceps. At the end of the procedure, pleuroscope and trocar were removed and a chest tube was inserted trough the chest wall.

Tissue samples from patients were collected after informed consent following approval of the ethical considerations by the Institutional Board of the University Hospital of Trieste, Italy.

Cell Isolation and Culture

Mesothelioma cells were isolated from pleural biopsy specimens. The tissue was finely minced with a cutter, incubated with a digestion solution composed of 0.5% trypsin (Sigma-Aldrich, Milan, Italy) and 50 μg/ml DNase I (Roche, Milan, Italy) in Hanks' Balanced Salt solution with $Ca^{2+}Mg^{2+}$ 0.5 mM (Sigma-Aldrich) overnight at 4°C. Next, the enzymatic solution was replaced with collagenase type 1 (3 mg/ml) (Worthington Biochemical Corporation, DBA) diluted in Medium 199 with Hank's salts (Euroclone Spa, Milan, Italy) for 30 min at 37°C. The digestion was blocked with 10% fetal bovine serum (FBS, GIBCO, Life Technology) and the cell suspension was filtered through a 100 μm pore filter (BD Biosciences, Italy).

The cells were seeded in a 12.5 cm^2 flask and cultured using Roswell Park Memorial Institute (RPMI) medium 1640 with GlutaMAX (Life Technologies, Milan, Italy), 45% human endothelial cells serum-free medium (HESF, Life Technologies), 10% heat-inactivated FBS supplemented with EGF (5 ng/ml), basic FGF (10 ng/ml), and penicillin–streptomycin (Sigma-Aldrich). Fresh medium was replaced every 2–3 days. MES were used at their five to eight passages for all the *in vitro* experiments.

Met5A cells were purchased from ATCC. These cells were grown in DMEM supplemented with 10% FBS and 1% antibiotic mixture (Sigma-Aldrich) and maintained at 37°C in humidified atmosphere with 5% CO_2.

Pleural Effusions

Malignant pleural effusions (MPEs) were obtained from patients who underwent thoracentesis after diagnosis of MPM in order to remove the exudative liquid filling pleural space. The surgery was performed within the Department of Pathologic Anatomy of the Hospital of Monfalcone (Gorizia, Italy). MPEs were immediately stored at 4°C for a maximum of 24 h before being processed. Approximately 1 Liter of MPE was centrifuged twice at 250 *g* for 10 min to remove the cells.

Dose-Determination of Soluble C1q

A 96-well plate (Corning Costar) was coated with sheep anti-C1q (1:6.000) diluted in carbonate/bicarbonate buffer (100 mM, pH > 9) and incubated overnight at 4°C. In order to avoid non-specific binding, the microtite wells were blocked with 2% skimmed milk (SM) in PBS and incubated for 2 h at 37°C. In the meanwhile, samples to be dose-titrated were prepared and then added to the wells in triplicate. A standard curve was prepared using a serial dilution of purified C1q (Sigma-Aldrich) from 50 to 1.56 ng/ml and the plate was incubated overnight at 4°C. Rabbit anti-human C1q (1:1,000) diluted in PBS + 0.5% SM + 0.05% Tween was incubated for 1 h at 37°C, followed by secondary probing with anti-rabbit IgG-alkaline phosphatase (AP) conjugate (1:20,000) for 30 min at 37°C. *p*-nitrophenyl phosphate (pNPP) was used as substrate, as described above, and the developed color was measured at 405 nm using the Titertek Multiskan ELISA Reader (Flow Labs).

Immunohistochemical Analysis

Tissue samples of different MPM histotypes were fixed in 10% buffered formalin and paraffin embedded. Sections of 4 μm in thickness were fixed with xylene, 100% EtOH, and 95% EtOH and then microwaved three times in Tris–HCl/EDTA pH 9.0 buffer (Dako, Milan, Italy) for 5 min and washed in Tris-buffered saline. After neutralization of the endogenous peroxidase with H_2O_2 (hydrogen peroxide) for 10 min, the sections were first incubated with PBS + 2% w/v BSA + 0.4% w/v Casein for 5 min in order to block the non-specific sites, and then probed with rabbit anti-human C1q (1:500) overnight at 4°C. The bound antibodies were revealed using the Vectastain Elite ABC horseradish peroxidase (HRP) kit (Vector Laboratories, DBA, Italy). Secondary antibodies were detected by 3-amino-9-ethylcarbazole (AEC) + high sensitivity Chromogen (Dako). The sections were counterstained with hematoxylin (Dako). Slides were examined under a Leica DM 3000 optical microscope and images were collected using a Leica DFC320 digital camera (Leica Microsystems, Wetzlar, Germany).

Alcian Blue Staining

After deparaffinizing and rehydrating, the sections were incubated with a solution of 1% Alcian blue dissolved in 3% Acetic acid, pH 2.5, for 30 min at RT. After washing in tap water for 10 min, the sections were dehydrated well in absolute alcohol and mounted. Images were acquired by the fluorescence microscope Leica DM 3000 using the Leica DFC320 camera.

Immunofluorescence Microscopy of MES

Mesothelioma cells cultured at confluence in an eight-chamber slide (BD Falcon) were fixed with FIX&PERM kit (Invitrogen, Life Technologies) for 15 min at RT. Incubation with primary antibodies (as listed earlier) was carried out for 1 h at RT. Cells were then washed and incubated with corresponding secondary antibodies (1:300) for 45 min at RT. The nuclei were stained with DAPI (Sigma-Aldrich). The glass was mounted with the Fluorescence Mounting Medium (Dako) and covered with a cover slip. Images were acquired by the fluorescence microscope Leica DM 3000 using the Leica DFC320 camera.

Flow Cytometry

Mesothelioma cells (5×10^5) were fixed with the fixation reagent FIX&PERM kit for 15 min at RT in dark and incubated with primary antibodies for 1 h at 37°C in a thermomixer (Eppendorf) at 800 rpm. Antibodies directed against intracellular antigens were diluted in permeabilization reagent of the FIX&PERM kit, while antibodies for cell surface antigens were diluted in PBS-1% w/v BSA. Incubation with secondary antibodies anti-mouse-FITC F(ab)′ (1:50) or anti-rabbit-FITC (1:300) was performed for 30 min on ice. Cells were suspended in 1% paraformaldehyde, the

fluorescence was acquired with the FACScalibur (BD Bioscience), and data processed using the software CellQuest.

Binding of C1q to MES

Mesothelioma cells were grown to confluence in 96-well tissue culture plates and incubated directly with increasing concentrations of purified human C1q or preincubated with increasing concentrations of HA, for 1 h at room temperature. Bound C1q was revealed by ELISA using mAb anti-human C1q (10 µl/ml) and alkaline-phosphatase-conjugated secondary antibodies (Sigma-Aldrich). The color, developed using pNPP (Sigma-Aldrich; 1 mg/ml) as a substrate, was read at 405 nm using a Titertek Multiskan ELISA reader (Flow Labs, Milano, Italy).

Coating Conditions

The microtiter wells were coated overnight at 4°C with HMW HA (50 µg/ml), C1q (20 µg/ml), ghA, ghB, ghC, or BSA (as a negative control; Sigma-Aldrich) diluted in 100 mM carbonate/bicarbonate buffer, pH 9.6. C1q was allowed to bind (25 µg/ml) to HA in PBS + 0.5% BSA, 0.7 mM $CaCl_2$, and 0.7 mM $MgCl_2$, overnight at 4°C.

Adhesion Assay

1×10^5 mesothelial cells or MES, labeled with the fluorescent dye FAST DiI (Molecular Probes, Invitrogen), were re-suspended in HESF (Life Technologies) containing 0.1% BSA (HESF + 0.1% BSA; Sigma), preincubated with 10 µM of ERK (#SCH772984, Selleckchem), JNK (#SP600125, SIGMA-Aldrich), or p38 (#SB203580, Selleckchem) inhibitors for 30 min at RT and then added to a 96-well plate (wells were coated as described above) for 35 min at 37°C in 5% v/v CO_2 incubator. Then, the unbound cells were removed and the adherent cells were lysed with 10 mM Tris–HCl, pH 7.4 + 0.1% v/v SDS. The plate was read *via* Infinite200 (544 nm, emission 590 nm) (TECAN) using a calibration curve generated through an increasing number of labeled cells.

Cell Proliferation

The cell proliferation assay was performed using Click-iT® Plus EdU Proliferation Kits (ThermoFisher). 5×10^3 MES were re-suspended in HESF + 0.1% BSA medium and seeded to a 96-well plate, which was earlier coated with C1q, HA or C1q + HA, as described above. Following adhesion, cells were incubated with 15 µM analog EdU nucleotide (5-ethynyl-2-deoxyuridine) for DNA incorporation during replication. Cells were then incubated for 24 h at 37°C, fixed, and set up for a marking reaction by azide Oregon-Green 488. The signal amplification step included the incubation with antibody anti-Oregon-Green conjugated with HRP that reacts with the substrate, Amplx UtraRed, and produces a bright response that beams fluorescence around red. The fluorescence was analyzed by TECAN (Tecan, Milan, Italy) in the excitation/emission range of 535/595 nm.

Apoptosis

Mesothelioma cells grown without serum, were suspended in 0.1% w/v BSA in HEFS, and 2×10^4 cells/well were seeded on precoated plates. The cells were left to adhere for 1 h at 37°C, then incubated with 500 µM of H_2O_2 for 6 h, before adding 5 µM of CellEvent™ Caspase-3/7 Green Detection Reagent (Life Technologies), a fluorogenic substrate for activated caspases 3 and 7.

Migration Assay

FAST DiI-labeled (Molecular Probes, Invitrogen, 1:100) MES (2×10^5 cells) were resuspended in 0.1% w/v BSA in HEFS and added to the upper chamber of a transwell system. The cells were allowed to migrate through HTS FluoroBlok systems with polycarbonate membranes of 8 µm pore size (Falcon) coated on the lower side, as described above. The plate was read using Infinite200, as described above.

Scratch Assay

Confluent monolayers of MES (2×10^5) were seeded in HESF + 0.1% BSA medium in 24-well plate. A scratch was placed in the middle of the well with a sterile 200 µl pipette tip. Subsequently, HA (50 µg/ml) or HA + C1q (20 µg/ml) were added to the wells. Cells incubated with 10% v/v FBS and as negative control MES were cultured in HESF/BSA (0.1%) medium without stimuli. Images were acquired by phase-contrast microscope (Leica).

Phosphorylation of ERK, SAPK/JNK, and p38 in MES

Pathway analysis was performed as per the manufacturer's instructions of the PathScan® Intracellular Signaling Array Kit (Fluorescent Readout) (#7744; Cell Signaling Technology, EuroClone, Milan, Italy). Briefly, 24 h serum-starved MES (1.8×10^6 cells) were left to adhere to HA, or HA-bound-C1q, as described above for the indicated periods of time at 37°C. Then, the cells were washed with ice-cold 1× PBS and lysed in 1× ice-cold Cell Lysis buffer containing a cocktail of protease inhibitors (Roche Diagnostics). The Array Blocking Buffer was added to each well and incubated for 15 min at RT. Subsequently, an equal amount of total lysate (0.8 mg/ml) was added to each well and incubated for 2 h at RT. After washing, the biotinylated detection antibody cocktail was added to each well and incubated for 1 h at RT. Streptavidin-conjugated DyLight 680 was added to each well and incubated for 30 min at RT. Fluorescence readout was acquired using the LI-COR Biosciences Infrared Odyssey imaging system (Millennium science) and data processed by the software Image studio 5.0.

Detection of the Interaction between Human C1q (and Its Recombinant Globular Head Modules) and HA

The microtiter wells were coated overnight at 4°C with either 50 µg/ml HA of different MWs diluted in carbonate/bicarbonate buffer (100 mM, pH 9.6). The blocking step with PBS + 1% BSA (2 h at 37°C) was followed by incubation with an increasing concentration of human C1q or the recombinant globular head modules (ghA, ghB, and ghC) of human C1q in PBS-$Ca^{2+}Mg^{2+}$ (0.7 mM) containing 0.5% BSA (PBS-CaMg-0.5% BSA) overnight at 4°C. Bound C1q was detected with sheep anti-human C1q polyclonal antibodies whereas bound ghA, ghB, and ghC were detected with mouse anti-MBP (1 h at 37°C). The binding

of anti-C1q was detected using anti-goat IgG-AP conjugate, whereas the anti-MBP binding was detected using anti-mouse IgG-AP conjugate for 30 min at 37°C. The phosphatase substrate, pNPP (Sigma-Aldrich) was dissolved in 0.1 M *glycine buffer* containing 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4 at a concentration of 1.5 mg/ml. The absorbance was measured at 405 nm with the Titertek Multiskan ELISA Reader (Flow Labs).

Evaluation of Complement Activation

Microtitre wells in a 96-well plate were coated with HA (50 µg/ml) or IgG (10 µg/ml), as described above. Non-specific binding sites were blocked with PBS-1%BSA for 1 h at 37°C, and then incubated with 20 and 50 µg/ml C1q diluted in PBS-CaMg-0.5%BSA for 1 h at 37°C. Subsequently, the wells were incubated with pooled normal human serum (1:100) as a source of complement components in PBS-CaMg-0.5%BSA and incubated for 30 min at 37°C with gentle shaking. C9 neoantigen detection was performed using the murine mAb aE11 against C9 neoantigen (kindly provided by Prof. T. E. Mollnes, Oslo, Norway) and incubated for 1 h at 37°C. The AP-conjugated anti-goat IgG (Sigma-Aldrich) or anti-mouse IgG (Sigma-Aldrich), used as secondary antibodies, were incubated 30 min at 37°C. pNPP (Sigma) was dissolved in glycine buffer at the concentration of 1.5 mg/ml. The absorbance was measured at 405 nm with the Titertek Multiskan ELISA Reader (Flow Labs).

Statistical Analysis

Data were analyzed using Two-way ANOVA, Tukey–Kramer test, and unpaired two-tailed Student's *t*-test or one-way ANOVA with Bonferroni corrections. Results were expressed as mean ± SEM. Non-parametric data were assessed by Mann–Whitney *U* tests and the results were expressed as median and interquartile range. p values < 0.05 were considered significant. All statistical analyses were performed using Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

C1q Is Present in Malignant Pleural Mesothelioma Specimens

We initially looked for the presence of C1q in a panel of invasive MPM specimens, including epithelioid, biphasic, and sarcomatoid (**Figure 1A**) histotypes. As shown in **Figure 1**, a strong positivity for C1q was detected in all tumor specimen types examined, particularly in epithelioid histotype.

Within the mesothelioma microenvironment, C1q was mainly expressed by monocytoid cells suggestive of tumor-infiltrating myeloid elements (shown by arrows in the upper panels) and in the small vessels, as indicated by the black triangles of the lower panels in **Figure 1B**. C1q was also diffusely present in the tumor stroma and associated with the cell membrane of tumor cells. Immunohistochemical controls of C1q are shown in **Figure 2**. The presence of C1q was also detected in the three pleural exudate by a quantitative ELISA assay. We found out that the concentration of C1q was found to be about 76 µg/ml (±8 µg/ml), approximately two- or threefold lower than our control serum (about 200 µg/ml).

Binding of C1q to HA does not Activate the Complement Classical Pathway

Having shown that C1q is abundantly present in the mesothelioma microenvironment, we investigated the ability of C1q to interact with ECM components. We focused our attention to HA which is abundantly present in mesothelioma tissue (22). The presence of HA is clearly evident in **Figure 3**, where we show sections obtained from epithelioid and a biphasic mesothelioma tissue stained with Alcian blue.

We previously demonstrated that C1q is able to bind to a range of target ligands present in the ECM; this interaction is particularly strong with HA (17). We confirmed by ELISA the ability of C1q to bind to HA in a manner similar to IgG (**Figure 4A**). We also analyzed the binding of C1q to HA of different molecular weights (**Figure 4B**). Our results indicated that there were no statistically significant differences in C1q binding to low and high MW-HA. Furthermore, we evaluated the capability of HA-bound C1q to activate the complement classical pathway, by measuring the C4 and C9 (neoantigen) (C9 neo) deposition by ELISA. As shown in **Figures 4C,D**, only C1q bound to IgG, and not HA-bound C1q, induced complement activation, and therefore, C4 deposition and C9-neoantigen formation. In order to localize the C1q interaction with HA, we analyzed the binding of recombinant forms of individual globular head modules (ghA, ghB, and ghC) to HA. Our results indicated that the globular head of C1q A chain (ghA) bound to HA better than ghB and ghC (**Figure 4E**), suggesting a differential and modular nature of the interaction between the gC1q domains and HA.

Isolation and Characterization of Primary Tumor Cells from MPM Biopsies

Having established that C1q is present in the MPM microenvironment and that it can bind to HA, we sought to investigate the implication of its presence in MPM biology. Therefore, we isolated MES from a portion of the resected malignant pleura, obtained during diagnostic pleuroscopy, from five patients with epithelioid MPM. We compared the MES morphology with Met5A, a commercial, immortalized mesothelial cell line, commonly used as a model of healthy cells. MES had mainly an elongated and filamentous shape and were very heterogeneous and multishaped (**Figure 5A**). It is possible to notice that there are also some polygonal and more regular cells in culture, which seem to resemble the epithelial phenotype of Met5A. Generally, they had an abundant cytoplasm in which vacuoles or granules were often present, transforming themselves in signet-ring cells.

We characterized MES for the expression of typical mesothelial markers, such as mesothelin, calretinin, CK8-18, and CD44 by immunofluorescence microscopy. The cells were also positive for vimentin, a marker of mesenchymal cells (**Figure 5B**). MES were positive for the above-mentioned markers. To assess the

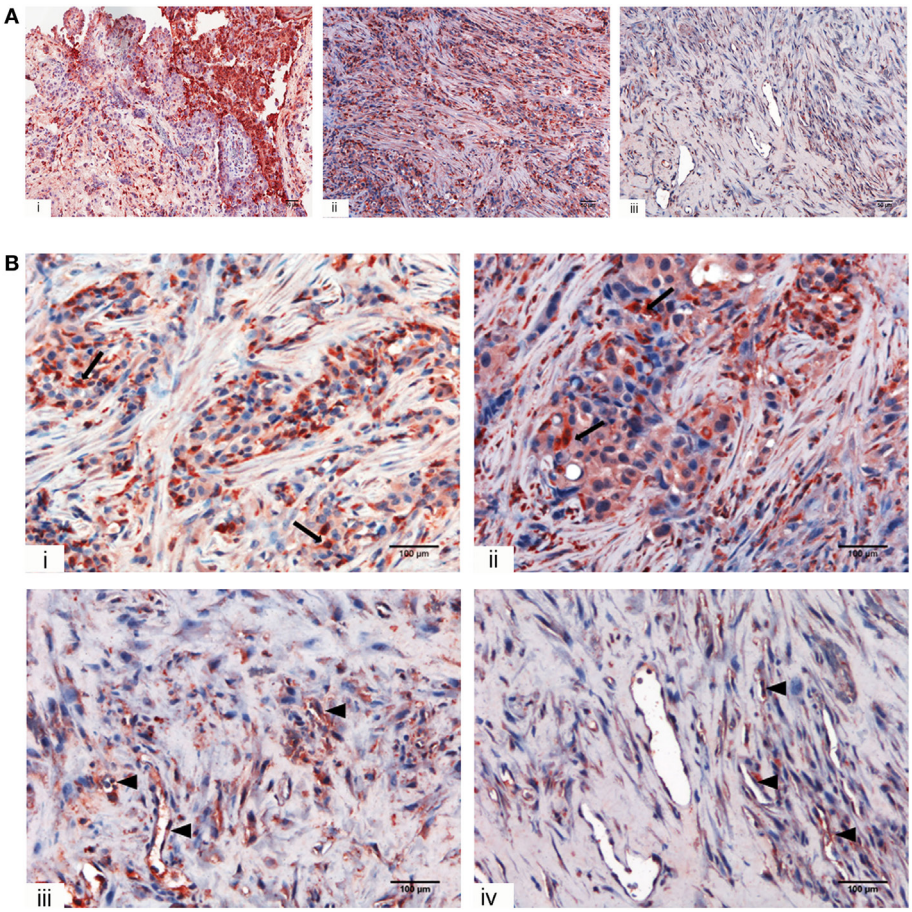

FIGURE 1 | Immunohistochemical analysis for C1q in human mesothelioma. **(A)** Representative microphotographs showing expression of C1q in different malignant pleural mesothelioma (MPM) histotypes: epithelioid (i), biphasic (ii), and desmoplastic (iii). Streptavidin–biotin–peroxidase complex system with 3-amino-9-ethylcarbazole (AEC) (red) chromogen; scale bars, 50 μm. **(B)** Representative microphotographs showing the expression of C1q in tumor-associated stroma of mesothelioma. Highlighted are monocytoid cells suggestive of tumor-infiltrating myeloid elements (arrows) and neovascular endothelial cells (arrow heads). Streptavidin–biotin–peroxidase complex system with AEC (red) chromogen; scale bars, 100 μm.

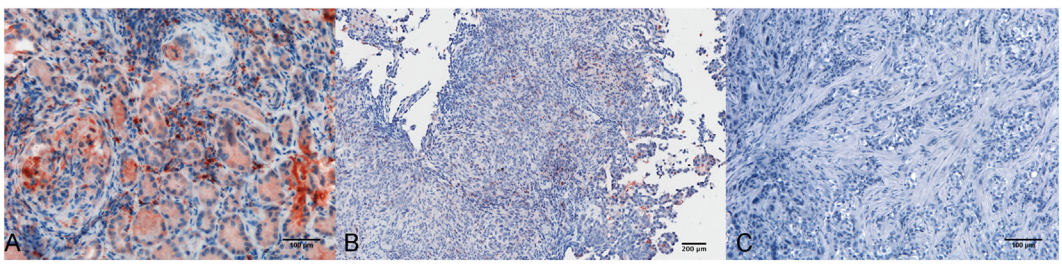

FIGURE 2 | Immunohistochemical controls for C1q. Representative microphotographs showing the immunohistochemistry controls: positive control tissue, lupus nephritis **(A)**, scale bar, 100 μm; negative control tissue, pleural benign cyst **(B)**, scale bar, 200 μm; negative control reaction, biphasic mesothelioma **(C)**, scale bars, 100 μm. Streptavidin–biotin–peroxidase complex system with 3-amino-9-ethylcarbazole (AEC) (red) chromogen.

purity of isolated primary cells, we performed immunophenotypical staining against the classical leukocyte antigen CD45 to avoid the presence of contaminating leukocytes. Furthermore, we also found that MES cells were negative for vWF, a common marker used to detect endothelial cells. Interestingly, all MES were positive for CD68, a mature macrophage marker, although they were negative for CD14. Some of these markers were also analyzed by FACS (**Figure 5C**; **Table 1**).

IHC-positive staining for C1q in MPM tissues suggested that C1q may be produced locally. In this regard, C1q expression

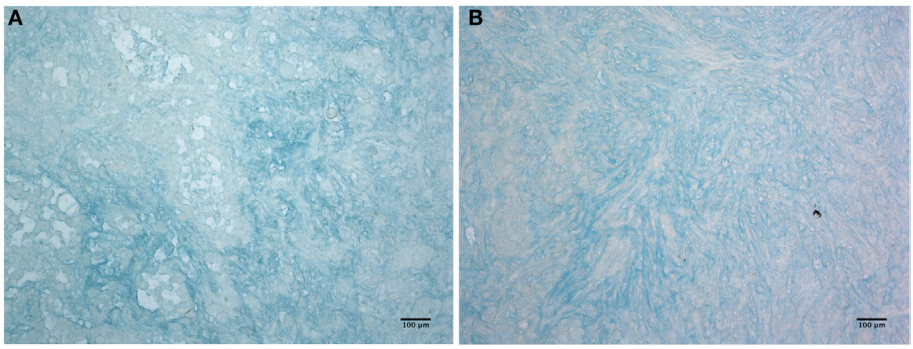

FIGURE 3 | Histochemistry of hyaluronic acid on malignant pleural mesothelioma (MPM). Alcian blue staining highlights hyaluronic acid of epithelioid **(A)** and biphasic **(B)** malignant mesothelioma sections. Scale bars, 100 µm.

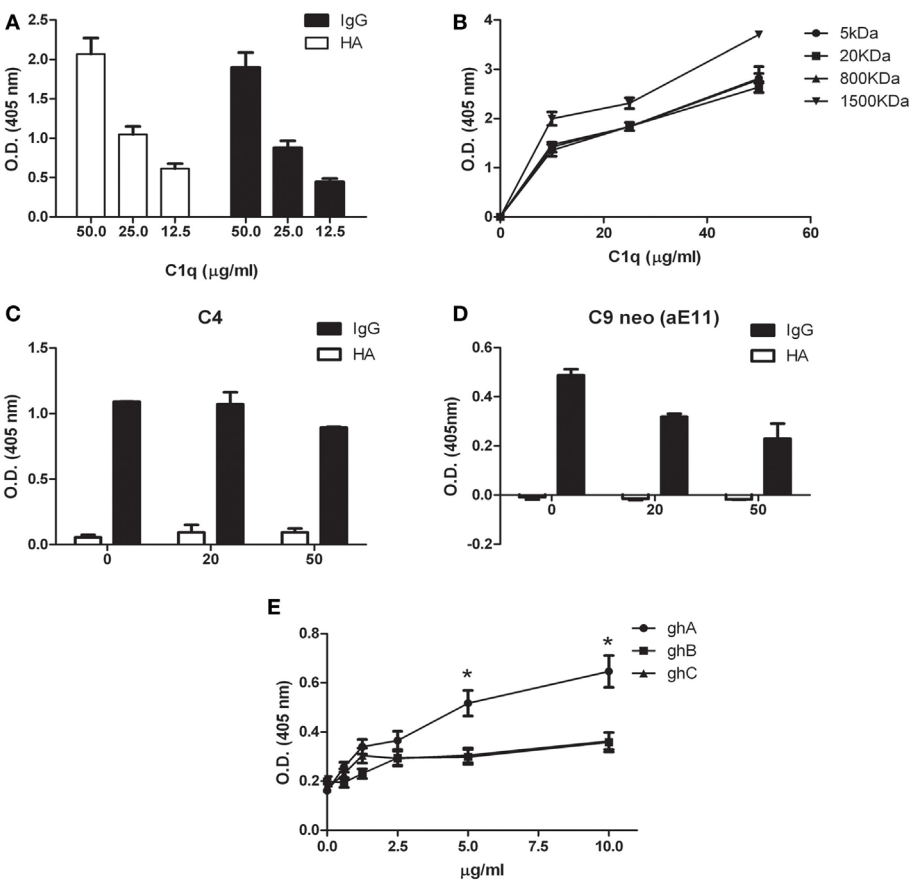

FIGURE 4 | Interaction of C1q with hyaluronic acid. **(A)** C1q interaction with high-molecular-weight (HMW) hyaluronic acid (HA) and IgG (positive control) by ELISA. Microtiter wells coated with 50 µg/ml HMW HA or 10 µg/ml IgG were incubated with an increasing concentration of C1q; HA-bound C1q was revealed with anti-C1q antibodies. C1q bound HA with an affinity similar to IgG. The data are expressed as mean of three independent experiments in triplicates ± SEM. **(B)** Binding of C1q to different MW HA. Microtiter wells coated with 50 µg/ml HMW-HA (or 5, 20, 800, or 1,500 kDa HA) were incubated with increasing concentration of C1q and bound C1q was revealed with anti-C1q antibodies. No statistically significant difference was observed between the binding of C1q to low and high MW-HA was observed. The data are expressed as mean of three independent experiments carried out in triplicates ± SEM. **(C–D)** Evaluation of the ability of C1q bound to HA to activate the classical pathway of the complement system. Microtiter wells coated with 50 µg/ml HMW HA, or 10 µg/ml IgG were incubated with 20 and 50 µg/ml C1q. Pool of normal human sera (1:100) was added as a source of complement components and the deposition of C4 **(C)** and the formation of C9-neoantigen (C9 neo) **(D)** were revealed by using specific antibodies against C4 and C9 neoantigen (mAb aE11) by ELISA. The data are expressed as mean of three independent experiments carried out in triplicates ± SEM. **(E)** Dose response curve of the binding of ghA, ghB, and ghC to HMW HA. Microtiter wells were coated with 50 µg/ml HMW HA. The wells were then incubated with increasing amounts (0–10 µg/ml) of recombinant C1q globular head modules (ghA, ghB, ghC). The binding was revealed using anti-MBP antibody. The data are expressed as mean of three independent experiments done in triplicates ± SEM.

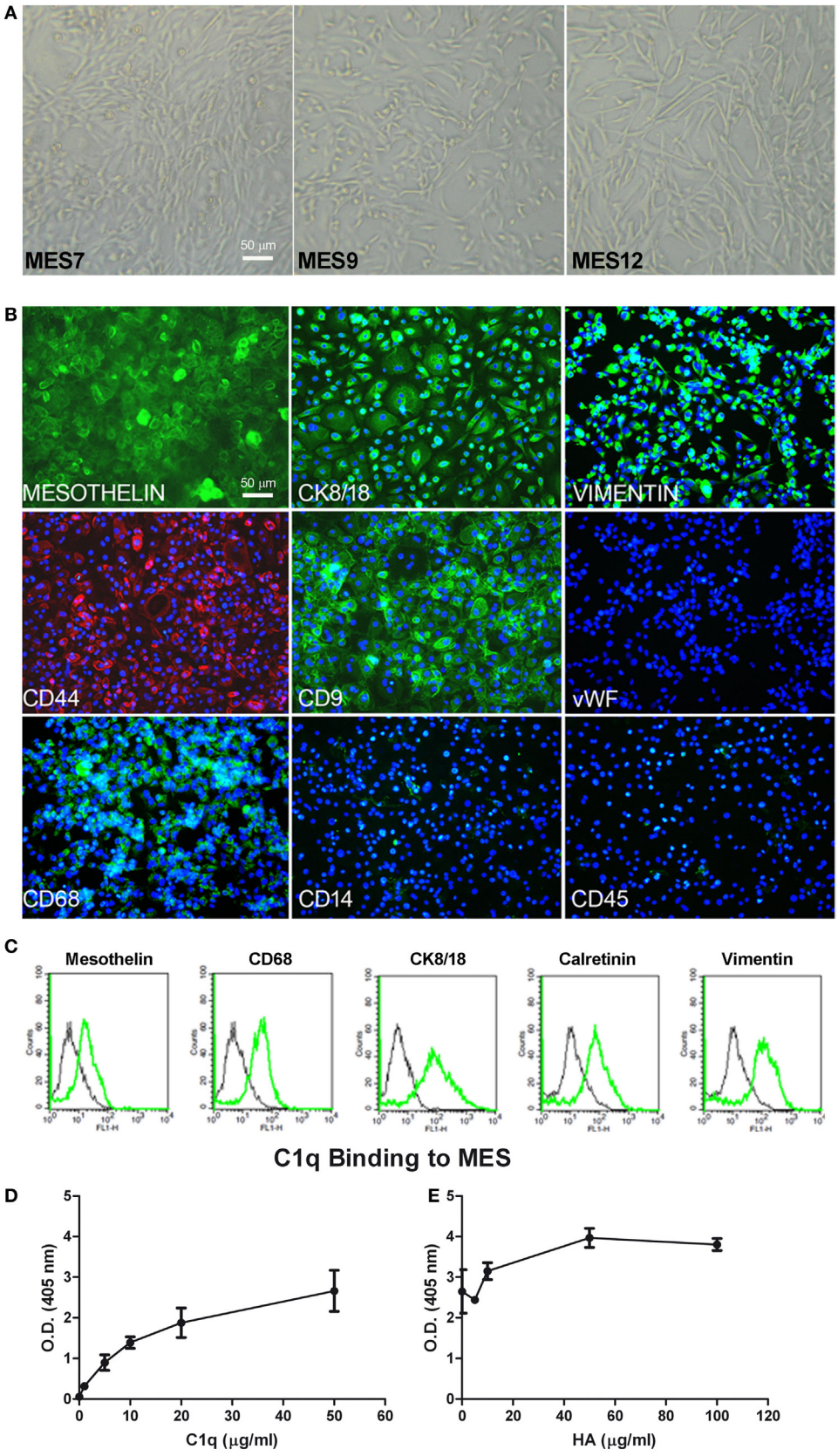

FIGURE 5 | Continued

FIGURE 5 | Continued
Characterization of mesothelioma cells (MES). **(A)** Morphological features of the three different populations of MES (MES7, MES9, and MES12) isolated from malignant pleural mesothelioma (MPM) biopsies. Images were acquired by phase-contrast microscope, Leica original magnification: 200×. **(B)** Mesothelial cells were characterized by immunofluorescence for the expression of mesothelin, CK 8/18, vimentin, CD9, CD68 (green), and CD44 (red). Mesothelioma cells were grown to confluence in eight-chamber culture slides. After fixation and permeabilization, the cells were stained with mAb anti-human mesothelin, CK 8/18, vimentin, CD9, von Willebrand factor (vWF), CD68, and CD14, followed by anti-mouse-FITC F(ab)′ secondary antibodies or mAb anti-human CD45 and mAb anti-human CD44-PE conjugate. Nuclei were stained blue by DAPI: original magnification 200×. **(C)** The expression of mesothelin, CD68, CK8/18, calretinin, and vimentin was confirmed by FACS. The expression of these markers (green lines) was compared with appropriate control antibodies (black lines). **(D)** Binding of C1q to MES**.** Tumor cells grown to confluence on 96-well tissue culture plates were incubated with increasing concentrations of purified C1q for 1 h at room temperature and the bound C1q was revealed by ELISA. **(E)** The binding of 25 μg/ml C1q to MES was detected preincubating the cells with increasing concentration of hyaluronic acid (HA). Bound C1q was revealed by ELISA as described above. The data are presented as mean ± SEM of three separate experiments.

TABLE 1 | Marker expression evaluated by FACS analysis on cells isolated from five different epithelioid mesothelioma tumors.

Marker	MES7	MES9	MES12	MES13	MES14
Mesothelin	+	+	+	+	+
Vimentin	++	++	+	+	+
CK 8/18	++	Nd	+	+	+
Calretinin	+	Nd	–	+	+
vWF	–	–	–	Nd	–
CD68	++	+	+	+	+
CD45	–	–	–	–	–
CD44	++	+	Nd	Nd	++

Data refer to cells between the third and the fifth passage in culture. ++ = positivity (>50%), + = positivity (<50%), – = negativity, Nd = not done.

was evaluated at both mRNA and protein levels in healthy (Met5A) and tumor MES. We performed qPCR for three C1q chains (*C1qA*, *C1qB*, and *C1qC*); none were positive in five different cell populations (data not shown). Interestingly, C1q was found to bind strongly to the surface of MES (**Figure 5D**) and the extent of binding increased in the presence of HA being highest at the concentration of 50 μg/ml of HA (**Figure 5E**). To investigate the role of bound C1q on complement activation, we incubated C1q-bearing cells with AB+ human serum and analyzed the cells for the presence of C4 and C9 neoantigen on their surface. We failed to detect both complement components suggesting that binding of C1q to MES is not associated with complement activation.

C1q Promotes MES Adhesion and Spreading

To evaluate the ability of C1q to interact with MES, we performed a cell adhesion assay using immobilized C1q. MES (four different populations) and Met5A cells were labeled with the fluorescent probe FAST DiI and seeded on to immobilised C1q, HA or HA-bound-C1q; BSA was used as a negative control protein. Met5A cells were able to adhere to HA, and to a less extent, to C1q or HA-bound-C1q (**Figure 6A**). In contrast, MES showed greater adherence to HA; HA-bound-C1q was able to enhance MES adhesion considerably compared to HA alone (**Figure 6B**). All four MES populations showed the same behavior on HA-bound-C1q, although MES adhesion to C1q alone varied considerably between patients' samples (**Figure 7**).

The analysis of the adherent MES by phase-contrast optical microscopy revealed that a high proportion of the cells seeded on to C1q or HA-bound-C1q were spread out, in contrast to the round morphology exhibited by those attached to HA or BSA (**Figure 6C**). In order to understand which module of the globular domain of C1q was mainly involved in MES adhesion, MES were stained with Fast DI and allowed to adhere to C1q, HA or recombinant globular heads (ghA, ghB, ghC) for 30 min. As shown in **Figure 6D**, the interaction of the cells with C1q seems to be mediated by ghC, and to a lesser extent, ghB. The cells did not seem to interact with ghA.

Since both HMW-HA and LMW-HA are present in the tumor microenvironment, we investigated the adhesion of MES to LMW-HA alone or in combination with C1q and we compared the results with the adhesion to HMW-HA with or without C1q. We did not observe any statistical difference between the MES adhesion to LMW or HMW-HA (**Figure 6E**).

C1q Promotes MPM Tumor Progression by Favoring MES Proliferation and Migration

We investigated whether C1q might contribute to tumor growth by stimulating the proliferation of MES. Thus, MES were seeded on to solid phase C1q, HA, or HA-bound-C1q and the number of proliferating cells were evaluated using the Click-iT® EdU Microplate Assay. Our results indicated that HA-bound C1q was able to enhance the proliferation rate of MES (**Figure 8A**), compared to HA or C1q alone.

C1q-induced migration of labeled MES was examined by monitoring cell migration from the upper chamber through an insert coated with C1q, or HA-bound-C1q. C1q (60%) was found to be more effective than HA (~20%), in effecting migration (**Figure 8B**). In this assay, we did not observe a synergistic effect of the double matrix (HA-bound-C1q). In fact, the percentage of the cell migration due to HA + C1q was comparable to that observed with C1q alone.

The effect of C1q on the cell migration was also analyzed using a scratch wound healing assay, monitoring the mobilization of MES toward the scratched area for 18 h. As shown in **Figure 9**, MES stimulated by C1q started to enter the scratched area and migrated farther than cells exposed to HA after 18 h.

In order to investigate whether C1q was able to protect MES from apoptosis, serum-starved MES were allowed to adhere on to the wells, coated with HA or HA-bound-C1q, and then incubated with 500 μM H_2O_2 for 6 h. Subsequently, the activation of caspases 3 and 7 was detected using a fluorogenic substrate. As shown in the graph in **Figure 8C**, the fluorogenic units of the MES on HA treated with H_2O_2 was double, compared to the untreated cells.

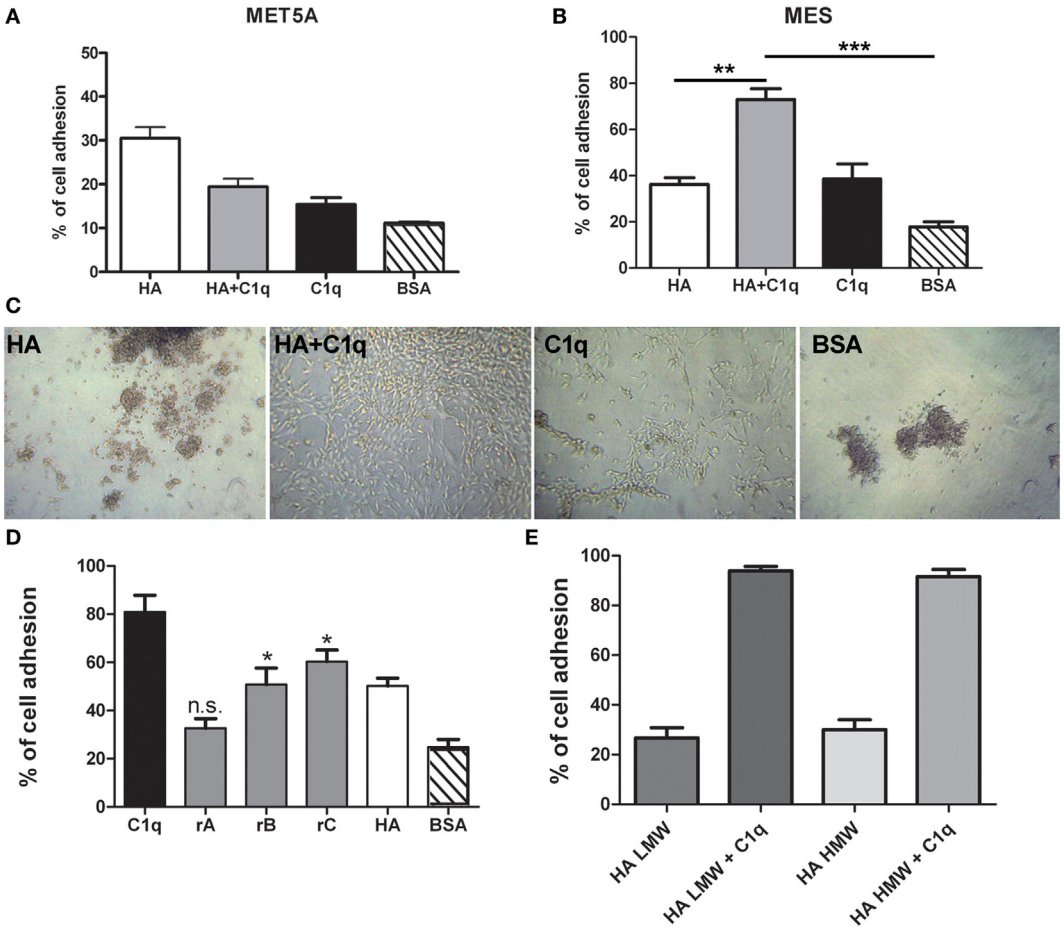

FIGURE 6 | Effect of C1q on mesothelioma cells (MES) adhesion. Mesothelial cells (Met5A) **(A)** and four different populations of MES **(B)** were labeled with the fluorescent dye FAST DiI and allowed to adhere to microtiter wells precoated with hyaluronic acid (HA), C1q, HA-bound-C1q, or bovine serum albumin (BSA). The data are expressed as mean of four independent experiments done in triplicates ± SEM. Cell adhesion was measured after 35 min. Results are expressed as percent of adhesion with reference to a standard curve established using an increasing number of labeled cells. **(C)** Morphological aspect of one representative primary cell line adhered to HA, C1q, HA-bound-C1q, or BSA. Images were acquired *via* phase-contrast microscopy, original magnification: 200×. **(D)** MES cells were labeled with the fluorescent dye FAST DiI and allowed to adhere to microtiter wells precoated with C1q, ghA, ghB, ghC, high molecular weight (HMW)-HA, or BSA. The data are expressed as mean of four independent experiments carried out in triplicates ± SEM. **(E)** MES were labeled with the fluorescent dye FAST DiI and allowed to adhere to microtiter wells precoated with HMW-HA, HMW-HA + C1q, low molecular weight (LMW)-HA, or LMW-HA + C1q. The data are expressed as mean of four independent experiments carried out in triplicates ± SEM.

Surprisingly, MES adhering to C1q or HA-bound-C1q were not protected from apoptosis induced by oxidative stress (**Figure 8C**).

C1q Enhances ERK1/2, SAPK/JNK, and p38 Phosphorylation in MES Cells

To further elucidate the mechanism of the cell activation, we analyzed the signaling pathways likely to be involved in tumor cell adhesion, migration and proliferation. We examined more specifically the activation of three members of the MAPK family, ERK1/2, SAPK/JNK, and P38 signaling molecules.

Serum-starved MES were allowed to adhere to wells coated with HA or HA-bound-C1q, for 5 or 20 min and the phosphorylation status of ERK1/2, SAPK/JNK, and P38 was evaluated by immunofluorescence using PathScan® Intracellular Signaling Array Kit (Cell Signaling Technology). As shown in **Figures 10A–C**, binding of MES to HA-bound-C1q resulted in the activation of all the signaling molecules, which was clearly seen at 20 min post stimulation for all pathways and the signal was significantly higher in the cells adhering to C1q bound to HA than in cells adherent to HA only. All three MAPK inhibitors tested in the adhesion assay proved to be effective in reducing significantly the cell adhesion to C1q bound to HA (**Figure 10D**).

DISCUSSION

C1q is expressed in the stroma and vascular endothelium of a number of human malignant tumors, including adenocarcinomas of colon, lung, breast, and pancreas. In a murine model of melanoma, C1q was found to promote cancer cell adhesion, migration and proliferation (18). Thus, as a pro-tumorigenic

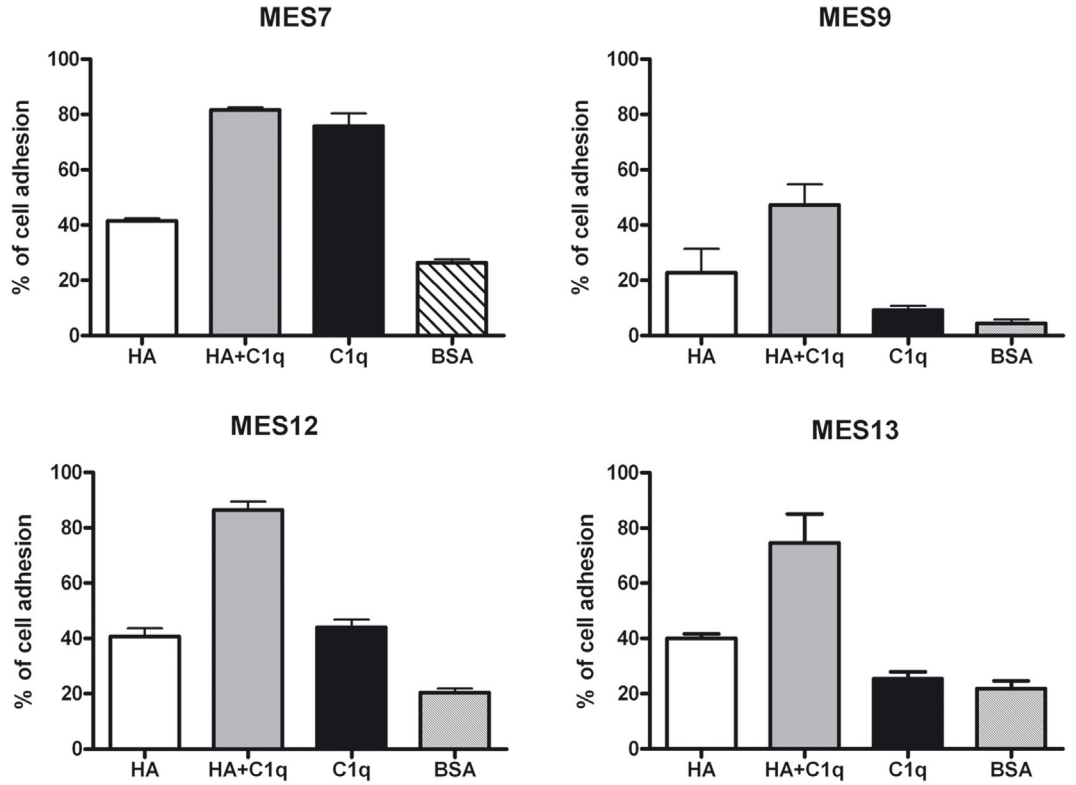

FIGURE 7 | Effects of C1q on mesothelioma cells (MES) adhesion. Four different populations of MES were labeled with the fluorescent dye FAST DiI and allowed to adhere to microtiter wells precoated with hyaluronic acid (HA), C1q, HA-bound-C1q, or bovine serum albumin (BSA). The results of each independent experiment, done in triplicates ± SEM are shown in order to highlight differential behavior of these cells. Cell adhesion was measured after 35 min. The results are expressed as percent of adhesion with reference to a standard curve established using an increasing number of labeled cells.

soluble factor, C1q can promote tumor progression by facilitating cancer cell seeding and angiogenesis. Here, we investigated the presence and the role of C1q in MPM for a number of reasons. MPM is an aggressive neoplasm with a poor prognosis, because it is resistant to chemotherapy and radiotherapy. Therefore, studying the microenvironment of this tumor can help devise novel therapeutic strategies. In this study, we particularly focused our attention on the interaction between C1q and HA and its implication on adhesion and proliferation of MPM cells. Understanding this C1q-HA interaction is of great importance given a unique expression pattern of C1q in mesothelioma tissues and a great relevance of HA in the biology of the MPM.

IHC revealed that C1q was present in all the histological variants examined and it seemed primarily associated with monocytoid cells, indicating that these cells might be the main source of C1q locally. C1q was also diffusely present in the tumor stroma and associated with the cell membrane of tumor cells mainly of the epithelioid histotype. This pattern of expression is similar to that in other solid tumors (18) and is reminiscent of its distribution in human decidua (17). There were also areas in which C1q could be found associated with small vessels, raising the possibility that C1q might exhibit a proangiogenic activity in this context, similar to that in wound healing (23).

We first investigated the interaction of purified human C1q with HA since MPM is associated with a high level of production of HA (24). HA is highly expressed in MPM because it is responsible for the lubrication of the pleural membranes and is secreted by the mesothelial cells (25). Here, we demonstrated that HA is abundantly present in MPM tissues, confirming an earlier study (22). HA has previously been shown to promote proliferation and migration of MPM cells through its interaction with hyaluronan receptor (26). We have previously shown that C1q can interact with HA (17). Here, we showed that C1q can bind to HMW-HA, and as expected, this binding does not induce complement activation. The interaction between C1 and HA has been studied in the past mainly considering its anti-complement activity (27, 28). The interaction of C1q with synovial HA in rheumatoid arthritis has been previously reported (29); however, C1q binds synovial antibodies that are covalently coupled to HA. Heated and then lyophilized HA binds C1q (and a range of complement components) better that native HA, probably *via* polyanionic charges (30). A comparable binding activity was also observed for LMW-HA, whose local accumulation can be detected in the tumor microenvironment as a consequence of enhanced synthesis and turnover of HMW-HA. Since LMW-HA, but not HMW-HA, can stimulate a number of biological processes (31, 32), it is likely that C1q can interfere with several functions

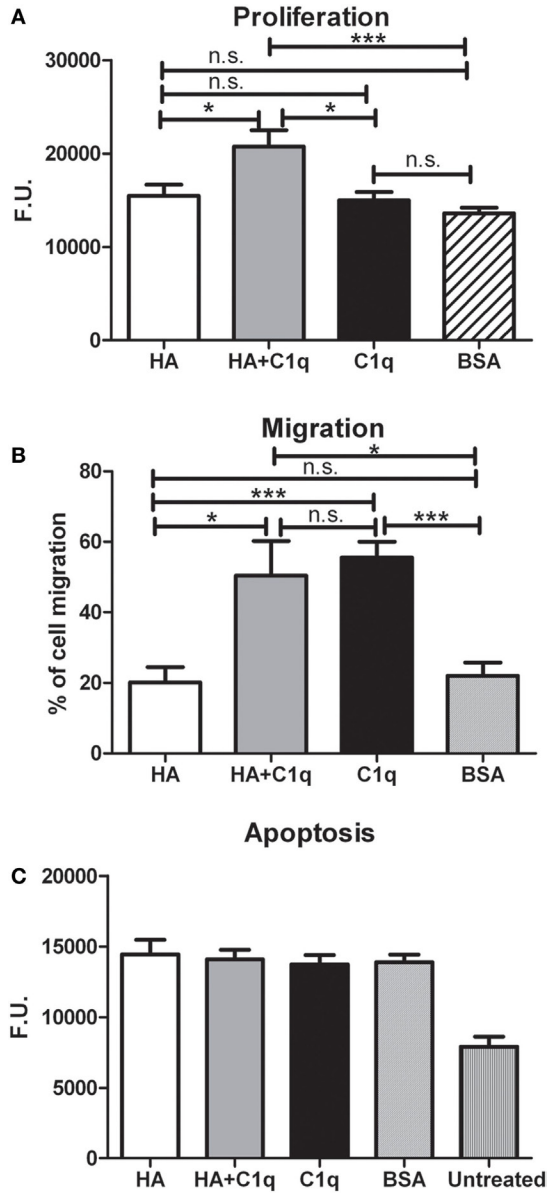

FIGURE 8 | Effects of C1q on mesothelioma cells (MES) migration, proliferation, and apoptosis. **(A–C)** The experiments of cell migration, proliferation and apoptosis were performed as detailed in Section "Materials and Methods." **(A)** MES proliferation. MES were seeded in wells precoated with hyaluronic acid (HA), C1q, HA-bound-C1q, or bovine serum albumin (BSA) and the number of proliferating cells was evaluated using the Click-iT® EdU Microplate Assay. **(B)** FAST DiI-labeled MES were allowed to migrate through a trans-well system polycarbonate membranes coated on the lower side with HA, HA-bound-C1q, C1q, or BSA. **(C)** Serum-starved MES cells were seeded in the wells precoated with HA, C1q, HA-bound-C1q, or BSA and incubated with H_2O_2. Apoptotic cells were ascertained by CellEvent™ Caspase-3/7 (Life Technologies). Data from at least five independent experiments are presented as mean ± SEM.

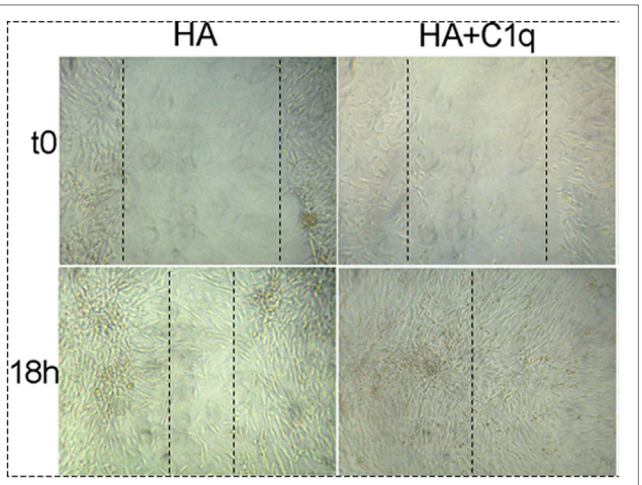

FIGURE 9 | Effects of C1q on mesothelioma cells (MES) migration. For wound healing assays MES (2×10^5) were seeded in HESF + 0.1% BSA medium in 24-well plate and grown to 90–95% confluence. A scratch was placed in the middle of the well with a sterile 200 µl pipette tip. Subsequently, hyaluronic acid (HA) (50 µg/ml) or HA + C1q (20 µg/ml) were added to the wells. The invasion was evaluated in three separate experiments performed independently. The results of a representative experiment are shown. Images were acquired by phase-contrast microscope, Leica original magnification: 200×.

mediated by HA, such as angiogenesis and inflammation. To dissect the functional contribution of each chain within the heterotrimeric globular domain of C1q, we investigated the binding properties of the recombinant ghA, ghB, and ghC modules. Our results demonstrated that the ghA module was the best binder of HA.

The proadhesive properties of C1q that we demonstrated *in vitro* for murine melanoma cells (18) were also evident for MES. In this study, we made a significant step forward since we were able to test several populations of MES isolated directly from human patients. Here, we demonstrated that the effects mediated by C1q-bound-HA were different from that observed for HA or C1q alone in terms of the number of adhering cells as well as morphology indicating that the presence of C1q can considerably modify the tumor microenvironment. The proadhesion effect of C1q seems to be restricted to the cells isolated from primary tumors while Met5A cells, which are representative of non-tumor mesothelium, adhere better to HA, compared to the adhesion on C1q-bound-HA or C1q alone. These data indicate that C1q can exert differential effects depending on the cell types and probably expression of putative receptors. Among various C1q receptors gC1qR, also called HA-binding protein-1, is an interesting molecule for its ability to bind both the gC1q domain of C1q and HA. Furthermore, CD44, the main receptor for HA, has been described as a possible docking signaling molecule for the interaction with gC1q (33). The nature and consequences of interaction between these cellular receptors and C1q-bound-HA is currently under investigation. Another interesting observation is that the adhesion of MES to C1q is predominantly mediated *via* the ghC module. Since ghA is the preferential binder to HA, the gC1q domain can act as a bridging molecule for anchoring the tumor cells to the ECM. C1q-bound-HA was able to promote the growth and the migration of MES *in vitro* confirming our previous results (18), obtained with B16/F10 murine cells and C1q bound to

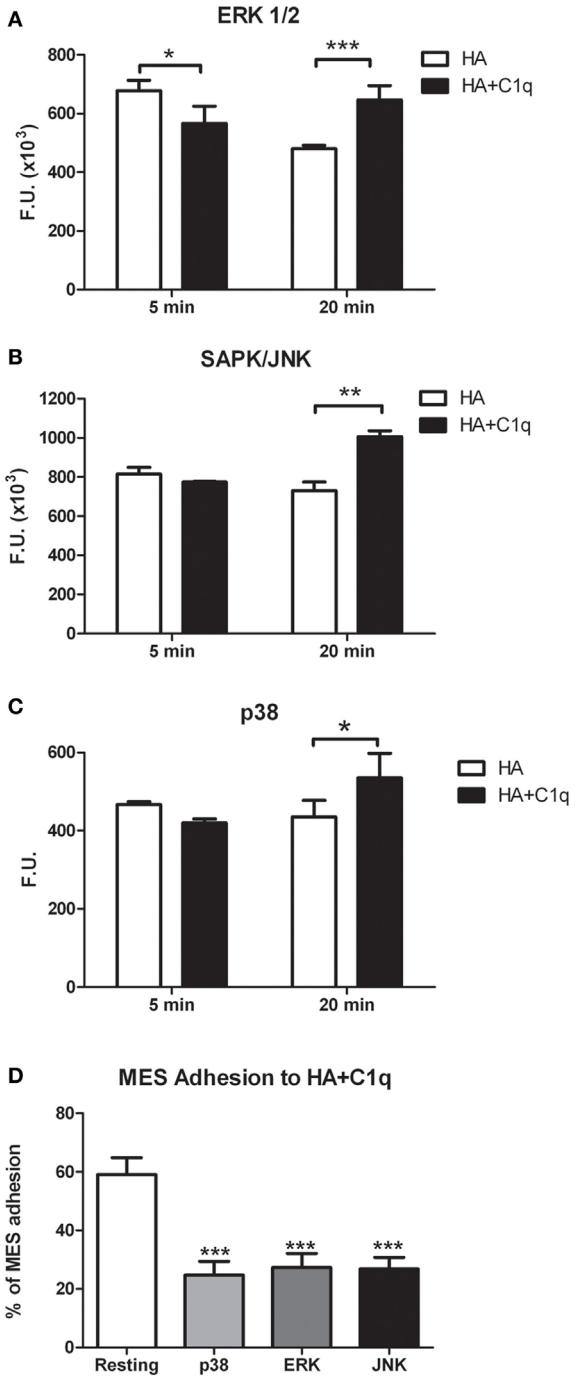

FIGURE 10 | Phosphorylation of ERK1/2, SAPK/JUNK, and p38 in mesothelioma cells (MES). **(A–C)** MES were allowed to adhere to hyaluronic acid (HA) or HA-bound-C1q and the phosphorylation status of ERK1/2, SAPK/JUNK, and p38 was evaluated using total cell lysates with PathScan Antibody Array Kit (Cell Signaling), as described in the Section "Materials and Methods." Data from at least three independent experiments are presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's *t*-test). **(D)** Three different populations of MES were labeled with the fluorescent dye FAST DiI, preincubated for 30 min at 37°C with P38, ERK, or JNK inhibitors and then allowed to adhere to microtiter wells precoated with HA-bound-C1q. The data are expressed as mean of three independent experiments done in triplicates ± SEM. $^{***}p < 0.001$ vs. resting.

fibronectin. The finding that C1q alone did not exert a significant proliferating effect on MES seen previously on melanoma cells may be explained by differential response of cells derived from various tumors to C1q. It is also important to emphasize that this study was carried out on primary cells, which were freshly isolated from patients.

The activation of three members of the MAPK family, ERK1/2, SAPK/JNK, and p38 in this study is also consistent with the previously reported study (17).

That C1q can act as a tumor promoting factor in MPM confirms our recent data (18). In addition, Winslow et al. have observed that the three chains of C1q were highly expressed in the stroma of breast cancers with poor prognosis (34). On the contrary, Hong et al. reported that C1q is involved in the regulation of cancer cell survival and progression sustaining the activation of the tumor suppressor *WW-domain containing oxidoreductase* (WOX1). C1q downregulation enhanced prostate hyperplasia and tumorigenesis because of the lack of WOX1 activation (35). Recently, Kaur et al. have reported that C1q, *via* its gC1q domain, induced apoptosis in an ovarian cancer cell line SKOV3 *via* TNF-α induced apoptosis pathway involving upregulation of Bax and Fas (36). These contrasting observations appear to suggest that the function of C1q in the biology of the tumor is complex and is strongly dependent on the microenvironment. Our hypothesis is that C1q can be locally produced by non-tumor cells and can interact differentially to the different ECM components present in the tumor microenvironment. The presence of C1q or C1 in soluble phase or bound to the ECM can provide different stimuli to the tumor cells present in the microenvironment.

In conclusion, C1q is abundantly present in mesothelioma tissue, interacts with HA, and interferes with adhesion, migration and proliferation of MES. The role of C1q is more complex than previously thought and is likely to be dependent on the tumor microenvironment. The availability of the recombinant globular domain of C1q may have implications for therapeutic approaches.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of governmental guidelines and approved by the CEUR (Comitato Etico Unico Regionale, FVG, Italy; number 34/2016), with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR NOTE

This work is dedicated to the memory of Bulla Gabriele and all mesothelioma patients.

AUTHOR CONTRIBUTIONS

Conception and design: RB, CA, and UK. Development of methodology: RV and LA. Acquisition of data: RV, VB, FZ, PG, and BB. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): CT, MC, AM, and BB.

Writing, review, and/or revision of the manuscript: RB, UK, CA, and FT. Study supervision: RB and MC.

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Prognostic Implications of the Complement Protein C1q in Gliomas

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The contribution of the complement system in the pathophysiology of brain cancers has been recently considered in light of its well-known involvement in carcinogenesis. Complement system represents an important component of the inflammatory response, which acts as a functional bridge between the innate and adaptive immune response. C1q, the first recognition subcomponent of the complement classical pathway, has recently been shown to be involved in a range of pathophysiological functions that are not dependent on complement activation. C1q is expressed in the microenvironment of various types of human tumors, including melanoma, prostate, mesothelioma, and ovarian cancers, where it can exert a protective or a harmful effect on cancer progression. Despite local synthesis of C1q in the central nervous system, the involvement of C1q in glioma pathogenesis has been poorly investigated. We, therefore, performed a bioinformatics analysis, using Oncomine dataset and UALCAN database in order to assess whether the expression of the genes encoding for the three chains of C1q (*C1qA*, *C1qB*, and *C1qC*) could serve as a potential prognostic marker for gliomas. The obtained results were then validated using an independent glioma cohort from the Chinese Glioma Genome Atlas datasets. Our bioinformatics analysis, coupled with immunohistochemistry and fluorescence microscopy, appears to suggest a positive correlation between higher levels of C1q expression and unfavorable prognosis in a diverse grade of gliomas.

Keywords: gliomas, C1q complement, bioinformatics analysis, survival probability, prognostic significance of C1q

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INTRODUCTION

The complement protein C1q represents the recognition subcomponent of the complement classical pathway, which is responsible for clearing immune complexes and invading pathogens. Its association with C1r and C1s, following ligand recognition, triggers complement activation (1, 2). C1q is characterized by a typical tulip-like overall structure, assembled from 18 polypeptide chains of three different types, A (28 kDa), B (25 kDa), and C (24 kDa), each having an N-terminal collagen-like domain and a C-terminal globular (gC1q) domain

(3, 4). The gC1q domain, which is the ligand recognition region of C1q, has a heterotrimeric structure, being composed of C-terminal ends of A, B, and C chains (5). In addition to binding IgG and IgM containing immune complexes and activating the complement classical pathway, there is emerging evidence to suggest that C1q plays crucial roles in several processes that are independent of complement activation, such as placentation (6), angiogenesis (7), autoimmunity (8, 9), and carcinogenesis (9, 10). C1q is highly expressed in the microenvironment of various types of human tumors (10, 11) where it can exert either a protective or a detrimental effect on the tumor growth. In prostate cancer cells, C1q was recently shown to induce apoptosis by activating the tumor suppressor WOX1 (12), thus acting as an anti-tumor humoral factor. In ovarian cancer, C1q has been shown to induce apoptosis in a representative SKOV3 cell line via activation of TNF-α, upregulation of Fas, and downregulation of mammalian target of rapamycin, RICTOR, and RAPTOR survival pathways (13). In a BALB-neuT mouse model of mammary carcinomas, C1q was shown to have a protective role against cancer progression (14). However, C1q can promote adhesion, migration, and proliferation of primary cells derived from malignant pleural mesothelioma patients, a relatively rare disease associated with exposure to asbestos (11). This dichotomous role of C1q has been further highlighted by a bioinformatics analysis involving several types of carcinomas (15).

The importance of C1q in the pathophysiology of the central nervous system (CNS) has been an area of intense research in the last two decades. In a healthy brain, C1q promotes synapse elimination required for fine circuitry refinement during CNS development (16). C1q activities, unrelated to complement activation, were shown to support neuronal survival and neurite outgrowth *in vitro* and protect against β-amyloid-mediated neurotoxicity (17). C1q can also interact with abnormal protein aggregates, such as βA1-42, thus favoring neurodegenerative diseases progression (18).

Since C1q and other complement components can be locally produced within the CNS by microglia and astrocytes, it is likely that C1q has involvement in primary brain tumor pathophysiology (19). Brain malignancies arise from cells of the CNS and are classified according to the tissue of phylogenetic origin. Gliomas represent the most common and aggressive form of brain tumors in adults; they are derived from glial or precursor cells (20). These are a heterogeneous group of diseases with multiple subtypes (20, 21). Glioblastoma multiforme (GBM) is the most common and fatal form of the primary brain tumor, accounting for approximately 60% of all glioma cases (22), whereas low-grade gliomas (LGGs) are the second most common type of glioma in adults (∼30%) (22).

In GBM tumor specimens, the presence of C1q does not correlate with CD45 positive leukocytic infiltration (23). Interestingly, C1q appeared to be highly concentrated around the malignant cells and the necrotic debris. Moreover, the serum concentration of C1q, together with critical components of the lectin and alternative complement pathways, appeared significantly increased in GBM patients as compared to healthy controls (23), thus indicating a role for complement activation in the pathogenesis of the GBM.

In the current study, we performed a bioinformatics analysis aimed at investigating whether C1q can serve as a potential prognostic marker for gliomas.

MATERIALS AND METHODS

Oncomine Database Analysis

The expression levels of *C1qA*, *C1qB*, and *C1qC* genes in gliomas were analyzed using Oncomine (www.oncomine.org), a cancer microarray database and web-based data mining platform for new discovery from genome-wide expression analyses (24, 25). We compared the differences in mRNA levels between normal tissue and cancer. The mRNA expression level in neoplastic tissues, compared to the healthy tissues, was obtained as the parameters of $P < 0.05$, fold change > 2, and gene ranking in the top 10%. Information about the datasets used in this study is summarized in **Table 1**.

UALCAN and CGGA Database Analysis

UALCAN (http://ualcan.path.uab.edu) is a web resource for analyzing cancer transcriptome data, which estimates the effect of gene expression level on the patient survival (26). In addition to the gene expression variation across tumor samples, gene-level correlations with patient survival also feature in UALCAN. Available genomics data from "The Cancer Genome Atlas" (TCGA) project was used for Kaplan–Meier survival analysis to generate survival probability plots (26). The prognostic significance of *C1qA*, *C1qB*, and *C1qC* expression and survival in gliomas were analyzed by UALCAN. The hazard ratio with 95% confidence intervals and logrank *p*-value were also computed.

The Chinese Glioma Genome Atlas (CGGA) (http://www.cgga.org.cn) is a user-friendly web application for data storage and analysis exploring brain tumors datasets from Chinese cohorts (**Table 1**) (27). Analyze tool of CGGA was used to browse *C1qA*, *C1qB*, and *C1qC* mRNA expression profile and to perform survival analysis in specific glioma subtype. The hazard ratio with 95% confidence intervals and logrank *p*-value were also computed.

Statistical Analysis

Survival curves were generated by UALCAN and CGGA. All results are displayed with *p*-values from a log-rank test. *P*-values <0.05 were considered significant. Similarly, in the case of Oncomine, the program provided the statistical significance of data (*P*-values).

Immunostaining

Tissue samples, derived from the neuroepithelial tumors with astrocytic differentiation, presenting different grades (grade-II and -III to GBM, grade-IV), were collected from glioma patients, after informed consent following approval of the ethical considerations by the Institutional Board of the University Hospital of Trieste, Italy.

Gliomas tissue specimens (five patients for each glioma grade, Department of human pathology of the University Hospital of Cattinara, Trieste, Italy) were fixed in 10% v/v buffered formalin and paraffin embedded. For immunostaining, 4 μm-thick tissue

TABLE 1 | Characteristics of the datasets used in bioinformatics analysis.

Datasets	Study description	Experiment type
Sun brain	One hundred fifty-seven (157) brain and CNS tumors and 23 normal brain samples were analyzed on Affymetrix U133 Plus 2.0 microarrays. Sample data includes type, grade, and sample name. Corresponding DNA copy number data is available in Kotliarov Brain	mRNA
French brain	Twenty-three (23) anaplastic oligodendroglioma, 4 anaplastic oligoastrocytoma, and 6 normal brain samples were analyzed on Affymetrix U133 Plus 2.0 microarrays. Sample data includes 10q loss of heterozygosity, 19q loss of heterozygosity, 1p loss of heterozygosity, age, chemotherapy response, EGFR amplification, sex, survival after diagnosis, survival after surgical resection, and therapy	mRNA
TCGA brain	Five hundred forty-seven (547) glioblastoma and 10 normal brain samples were analyzed. Sample data includes age, sex, survival, and others. This dataset consists of Level 2 (processed) data from the TCGA data portal. Corresponding DNA copy number data is available in TCGA Brain 2	mRNA
CGGA brain	The CGGA RNA sequence dataset consisted of 325 samples, including 109 grade-II samples, 72 grade-III samples and 144 grade-IV samples. Of the 144 GBM samples, 6 samples were lost to follow-up; therefore, 138 samples were included in the survival analysis. The patients with GBM were followed up every 3 months	mRNA
Rickman brain	Forty-five (45) astrocytoma and 6 normal temporal lobe samples were analyzed on Affymetrix HuGeneFL microarrays. Sample data include type and grade	mRNA
Bredel brain 2	Fifty (50) brain CNS carcinoma samples and 4 normal brain samples were analyzed on cDNA microarrays. Sample data includes disease type	mRNA
Liang brain	Thirty (29) glioblastoma, 3 mixed astrocytoma-oligodendroglioma, 2 oligodendroglioma, 2 normal brain, and 1 normal cerebellum sample were analyzed on cDNA microarrays. Sample data includes type, age, location, primary/recurrent, sex, and survival	mRNA

sections were de-waxed with two changes of xylene, 10 min each. Slides were then transferred to 100% alcohol, for two changes, 10 min each, and once through 95 and 70% alcohol respectively, for 5 min each. Finally, they were rinsed in de-ionized water, twice for 3 min each. The antigen unmasking technique was performed using Novocastra Epitope Retrieval Solutions pH9 EDTA-based buffer in thermostatic bath at 98°C for 30 min (28). Sections were brought to room temperature and washed in PBS. Subsequently, the neutralization of the endogenous peroxidase with 3% v/v H_2O_2 and Fc blocking by a specific protein block (Novocastra, Leica Biosystems) were performed.

For immunostaining, glioma sections were probed with the following primary antibodies overnight at 4°C: rabbit anti-human C1q polyclonal antibody (1:500; Dako), rabbit anti-human C3d polyclonal antibody (1:100; Cell Marque) and rabbit anti-human C4d polyclonal antibody (1:100; Cell Marque). Antibody-Antigen recognition was detected using Novolink Polymer Detection Systems (Novocastra Leica Biosystems, Newcastle) and employing the high sensitivity AEC (3-Amino-9-Ethylcarbazole) as chromogen. Slides were counterstained with Harris Haematoxylin (Novocastra, Ltd) and images were collected using a Leica DFC320 digital camera (Leica Microsystems, Wetzlar, Germany).

For double immunostaining experiments, tissue sections were incubated overnight at 4°C with the following primary antibodies: rabbit anti-human C1q polyclonal antibody (1:500, Dako), mouse anti-human CD68 (1:50, Clone KP1, Dako) and anti-human CD163 monoclonal antibodies (1:100, Clone 10D6, Leica Biosystems). The following secondary antibodies were used: goat anti-rabbit conjugated to Alexa Fluor 488, and goat anti-mouse conjugated to Alexa Fluor 568 (Life Technologies). Nuclei were counter-stained with DAPI (4′,6-diamidin-2-fenilindolo). All the sections were analyzed under Zeiss Axio Scope A1 optical microscope (Zeiss, Germany) and microphotographs were collected using an Axiocam 503 Color digital camera with the ZEN2 imaging software (Zeiss Germany).

RESULTS

Bioinformatics Analysis Reveals Higher mRNA Levels of *C1q A*, *B*, and *C* Chains in Gliomas

We initially compared the mRNA levels of the three chains of human *C1q* (*A*, *B*, and *C*) in normal brain and gliomas using the Oncomine platform. From the analysis performed on several datasets such as Sun's, French's, TCGA's, Rickman's, Bredel 2's, and Liang's, a significantly higher mRNA expression levels for *C1qA*, *C1qB*, and *C1qC* chains were detected in gliomas (different histotypes and grades) as compared to normal brain tissue (**Figure 1**, $P < 0.05$; **Table 2**).

We then took advantage of the UALCAN tool to carry out bioinformatics analysis on *C1qA*, *C1qB*, and *C1qC* mRNA expression levels according to TCGA database. UALCAN tool considers LLGs, grade-II and -III, and high-grade gliomas (HGGs) only grade-IV (or GBM) while the World Health Organization (WHO) considers as LLGs, grade-I and -II, and HGGs as grade-III and -IV. Based on this analysis, as shown in **Figure 2**, a positive correlation between the mRNA expression of the three chains and the unfavorable prognosis only in LGGs (grade-II and -III) was evident, where the survival probability is indeed reduced ($P < 0.05$). By contrast, no correlation was observed between *C1qA*, *C1qB*, and *C1qC* mRNA expression and the survival probability in GBMs (grade-IV) (**Figure 2**).

To further validate these results, we used the CGGA tool to inquire an independent glioma database. Based on this analysis, a positive correlation was found between the mRNA expression of the three C1q chains and the unfavorable prognosis in

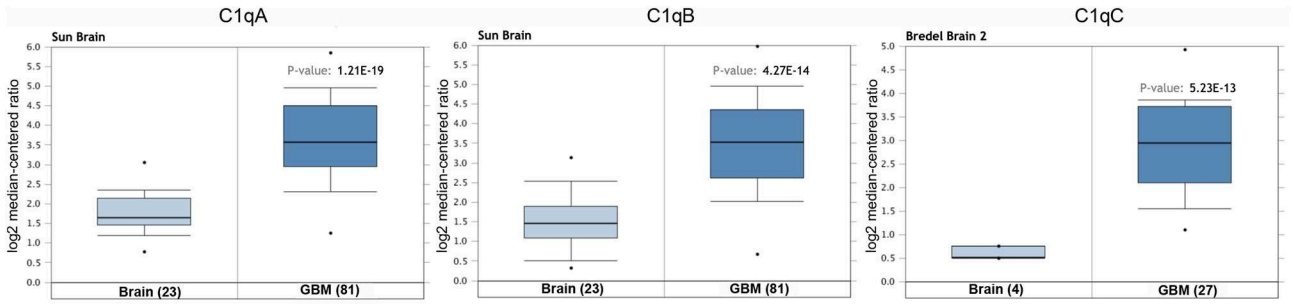

FIGURE 1 | *C1qA*, *C1qB*, and *C1qC* expression in gliomas. Sun's dataset was used for bioinformatics analysis to explore *C1qA* and *C1qB* mRNAs expression in the glioblastoma multiforme, whereas Bredel 2's dataset was used for bioinformatics analysis to evaluate *C1qC* mRNA. A higher expression of the three chains was detectable in glioblastoma multiforme compared to normal brain tissue. GBM, glioblastoma multiforme.

TABLE 2 | *C1qA*, *C1qB*, and *C1qC* expression in gliomas in the datasets used in the current study with Oncomine.

Datasets	C1q chains	Brain vs. tumor	*P*-value
Sun	*C1qA*	Brain (23) vs. Anaplastic astrocytoma (grade-III) (19)	3.59E-6
		Brain (23) vs. Glioblastoma multiforme (grade-IV) (81)	1.21E-19
		Brain (23) vs. Anaplastic astrocytoma (grade-III) (19)	5.22E-6
	C1qB	Brain (23) vs. Glioblastoma multiforme (grade-IV) (81)	4.27E-14
French	*C1qA*	Brain (6) vs. Anaplastic oligoastrocytoma (grade-III) (4)	8.89E-4
		Brain (6) vs. Anaplastic oligodendroglioma (grade-III) (23)	3.59E-6
	C1qB	Brain (6) vs. Anaplastic oligoastrocytoma (grade-III) (4)	5.89E-4
TCGA	*C1qA*	Brain (10) vs. Glioblastoma multiforme (grade-IV) (542)	1.63E-8
	C1qB	Brain (10) vs. Glioblastoma multiforme (grade-IV) (542)	3.45E-4
Richman	*C1qB*	Temporal lobe (10) vs. Astrocytoma (different grade) (45)	7.87E-4
Bredel 2	*C1qC*	Brain (4) vs. Glioblastoma multiforme (grade-IV) (28)	5.23E-13
Liang	*C1qC*	Brain (2) vs. Glioblastoma multiforme (grade-IV) (30)	2.12E-4

n, samples number.

all WHO grade of gliomas, where the survival probability is indeed reduced ($P < 0.05$) (**Figure 3**, lowest panels). A similar unfavorable prognostic effect was detected in grade-III gliomas ($P < 0.05$) while no correlation was observed between *C1qA*, *C1qB*, and *C1qC* mRNA expression and the survival probability in gliomas grade-II (**Figure 3**). In contrast to UALCAN analysis, a negative prognostic effect was underscored in GBMs ($P < 0.05$) (**Figure 3**).

Low- and High-Grade Gliomas Abundantly Express C1q Protein

We investigated the presence and the distribution of C1q in several glioma samples of different grades. As shown in **Figure 4**, a high expression level of C1q was observed both in LGGs as well as GBM.

Within the tumor-associated microenvironment, C1q was mainly expressed by monocytoid cells, suggestive of tumor associated macrophages (arrow heads) scattered among the neoplastic cells, which show an increased density around the intra-tumoral necrotic foci (**Figure 4C**). Moreover, the presence of C1q was detected in association with the vascular stroma; in GBM, it was also expressed in the vascular endothelial cells (**Figures 4E,F**). C1q deposition was not associated with complement activation, occurring either via the classical or the other pathways, since we failed to observe any C3d and C4d immunoreactivity in our glioma's specimens (**Figure 5**).

Infiltrating M2 Macrophages Are Likely Source of C1q in Gliomas

To further characterize the cell type infiltrating the tumor and actively involved in C1q synthesis, immunocytochemical experiments were performed on gliomas specimens via staining for C1q and CD68, a specific marker for monocyte/macrophage cell types. A clear co-localization of C1q/CD68 immunoreactivity was detected in both low- and high-grade gliomas (**Figure 6**), thus identifying the macrophages infiltrating glioma tumor as the main source of local C1q synthesis and secretion. Double labeling experiments were also performed using an anti-CD163, a specific marker for the tumor-promoting M2-polarized macrophages (**Figure 7**). Under these conditions, it appeared that not only the number of CD163 positive cells, but also the CD163 expression level itself, were increased in high-grade gliomas as compared to low-grade ones. Most of the C1q labeled cells colocalized with CD163, even though a small fraction of infiltrating cells were solely expressing C1q, suggesting that they may correspond to the CD68/C1q co-expressing M1-type of macrophages.

DISCUSSION

Glial tumors, also called gliomas, are the most prevalent form of adult brain tumor, accounting for nearly 80% of all brain malignancies (22). Based on their histological features and expression of lineage markers, gliomas can be classified into astrocytomas, oligodendrogliomas, ependymomas, and choroid

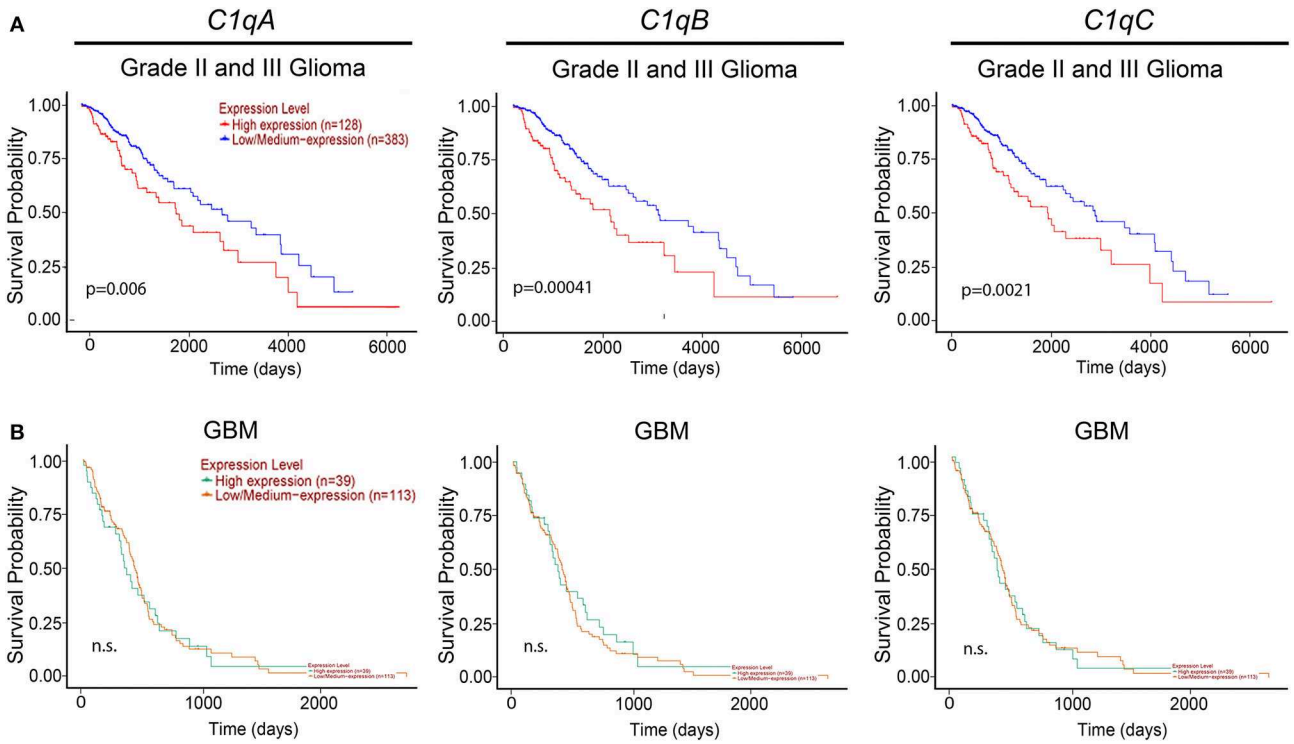

FIGURE 2 | Pathological significance of C1q expression in gliomas. According to the UALCAN database analysis, *C1qA*, *C1qB*, and *C1qC* mRNAs expression were negatively linked to a survival probability in patients with LGGs (grade-II and -III) **(A)**, whereas no correlation in patients with GBM (grade-IV) **(B)**. LGGs, low-grade gliomas; GBM, glioblastoma multiforme.

plexus tumors (29). Astrocytomas, which represent almost half of all primary brain and spinal cord tumors, may occur in the brain with a preferential localization in the cerebrum and affect mostly adults, particularly middle-aged men. In 2016, the WHO has redefined this classification scheme by introducing molecular parameters in addition to the well-established histopathological features (20, 21). These new guidelines have allowed classification of all pathological glial entities in four grades, according to histological parameters including nuclear atypia, mitoses, vascular proliferation, and necrosis. According to the grading system, astrocytic neoplasm can be divided into low and high-grade astrocytomas. Low-grade astrocytomas are the least malignant tumors characterized by slow growth and good prognosis being the *pilocytic astrocytomas* (grade-I) and *diffuse astrocytomas* (grade-II), the most frequent types. High-grade astrocytomas are glial tumors presenting a rapid growth with a tendency to infiltrate nearby brain tissues. They are divided into astrocytomas grade-III (*anaplastic astrocytoma*) and grade-IV (*glioblastoma multiforme*, *GBM*). GBM is the most aggressive and fast-growing malignancy characterized by poor clinical outcome. It can arise in the brain *de novo,* or it can evolve from lower-grade astrocytomas or oligodendrogliomas (29). Recent studies have begun to address the immune signature of the glioma microenvironment and its relationship with prognosis (30). The fact that C1q can be locally synthesized within the CNS and that it is involved in tumor immunology, we wanted to interrogate its relevance in the pathogenesis and prognosis of gliomas.

In the current study, we performed a bioinformatics analysis to unveil whether C1q could serve as a potential prognostic marker for these devastating malignancies. UALCAN queries of the TCGA and the CGGA datasets were analyzed to validate our initial result. It highlighted a significant correlation between high expression level of the three chains of the C1q and poor prognosis in gliomas of diverse grade of malignancy (**Figures 2**, **3**). In particular, while interrogating the CGGA dataset, which allowed distinction between grade-II and grade-III gliomas, a significant correlation was established only for grade-III gliomas (**Figure 3**). In the TCGA dataset, significance was achieved for the so-called LGGs, which combines grade-II and -III cases (**Figure 2**). The main contradiction was noticed in GBMs (grade-IV gliomas), where opposite prognostic effects were underscored in relation to the dataset used (**Figures 2**, **3**). One possible explanation for these contradictory observations may originate from the algorithm utilized by the bioinformatics web resources to define high and low expression profile of the genes. Another aspect to take into account is potential differences in the genetic and epigenetic signatures characterizing the two datasets, the TGCA mostly relying on Caucasian patients while CGGA relying on Chinese cohorts. Indeed, it has been proven that substantial variation in glioma incidence and survival are connected, to some extent, to race and

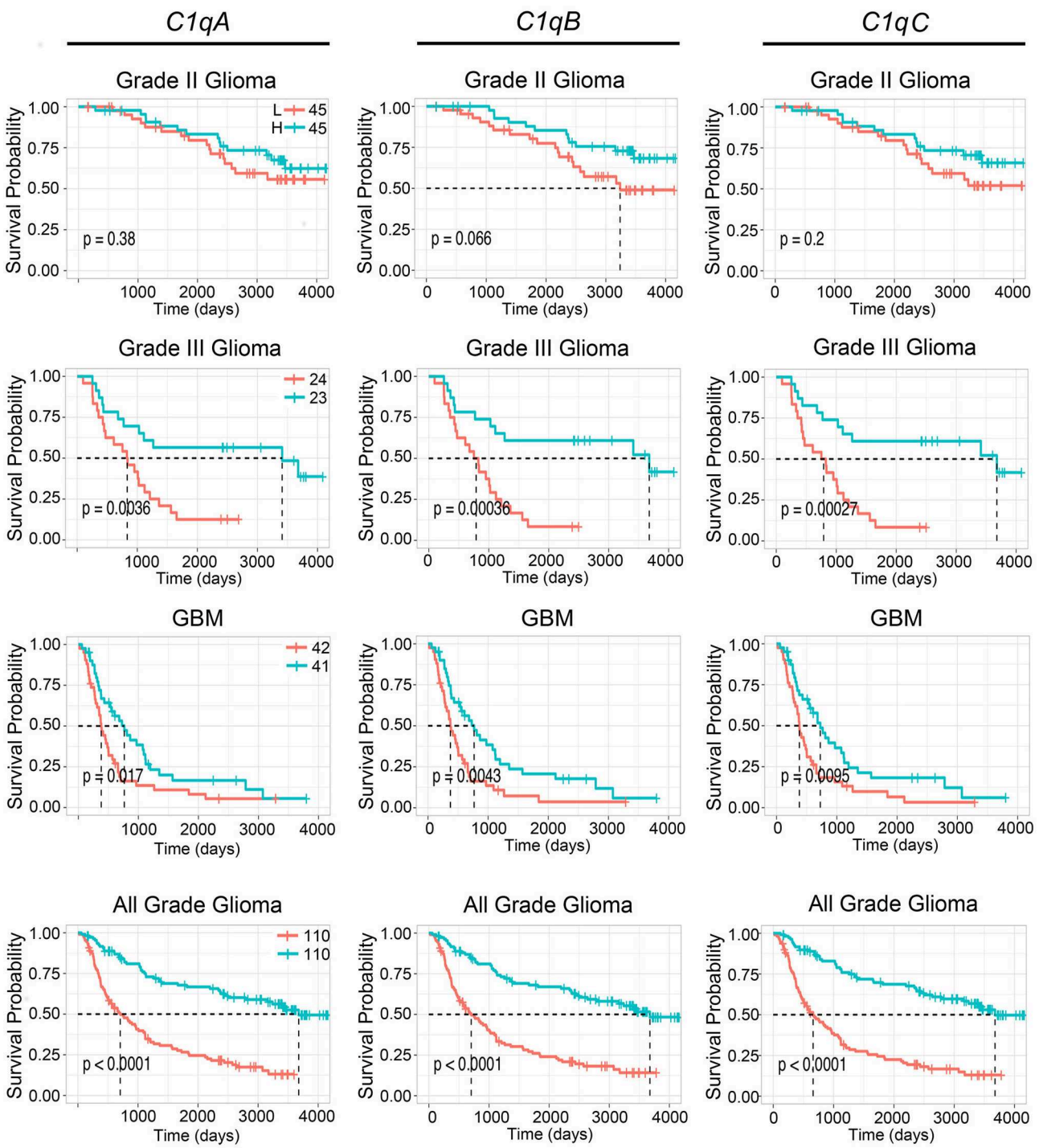

FIGURE 3 | Pathological significance of C1q expression in gliomas. According to the CGGA database analysis, *C1qA*, *C1qB*, and *C1qC* mRNAs expression were negatively linked to a survival probability in patients with grade-III and GBM. H, high expression; L, low expression.

ethnicity (31). Finally, it is worth mentioning that GBM exists in two forms, primary and secondary, indistinguishable histologically, but clearly discernible clinically and in terms of molecular signatures (29, 32). Primary GBM is the most common form that occurs mainly in adults over 50 years of age. The genetic profile is characterized by epidermal growth factor receptor (EGFR) overexpression, phosphatase and tensin homolog (PTEN) mutation, p16 deletion, and chromosome

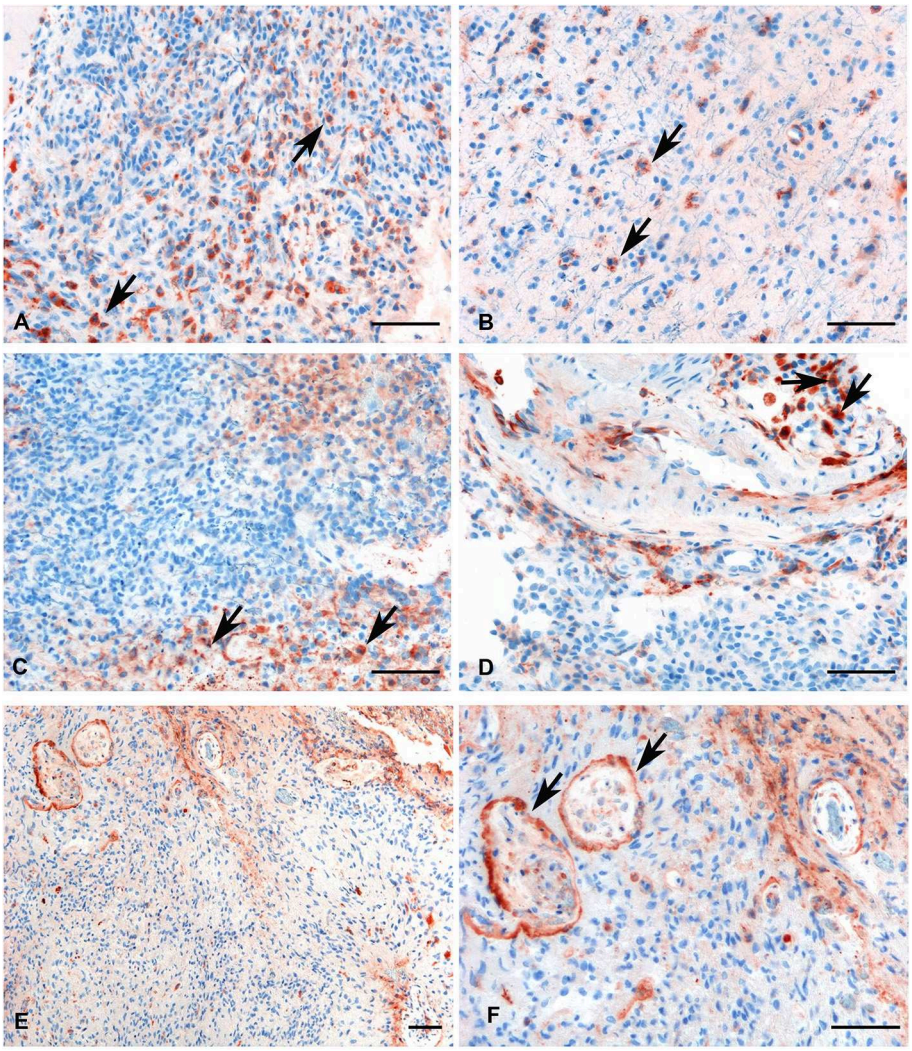

FIGURE 4 | Representative immunohistochemical analysis of C1q in low **(A,B)** and high grade **(C,D)** gliomas showing a high expression in both histotypes regardless of grade. C1q results mainly expressed by macrophages and the vascular stroma (see arrows). C1q expression in the endothelial cells is shown in panels **(E,F)** (see arrows, low, and high magnification). Polymer detection system with AEC (red) chromogen; scale bars, 50 μm.

10 loss (32). Secondary GBM derives from a malignant progression of diffuse or anaplastic astrocytomas (grade-II and -III, respectively), and occurs in younger patients, characterized by p53 mutation and a reduced state of heterozygosity in tumor cells (loss of heterozygosity) on chromosome 10q (32). Both datasets used in our bioinformatics analysis did not discriminate between *de novo* and secondary GBM, possibly masking a significant correlation, indeed expected, in those tumors progressing from grade-III gliomas.

There is emerging evidence to confirm that C1q is involved in cancer pathophysiology, being an important modulator of inflammation and cytokine/chemokine/growth factor secretion. What is still quite puzzling is whether and when C1q is protective against or supportive of cancer progression. On one hand, C1q can be detrimental to cancer cell viability via its cell lytic, anaphylatoxin, and opsonin effector mechanisms (33). Alternatively, C1q can exert tumor-promoting functions which are independent of the classical pathway activation. As observed for other types of cancers (10, 15), C1q deposition in gliomas seems not to be correlated with complement activation as we were unable to detect the complement split products C3d and C4d in the cohort of tumor specimens tested ($n = 5$). It is worth noting that our conclusions are based on the use of only five patients' tissue samples that were subjected to immunohistochemistry. This is in contrast to other cancers such as renal and lung cancers, where C4d deposition has been reported (34, 35). The mechanisms underlying such binary features of C1q are likely to be shaped by the type of cancer cells, nature and extent of infiltrating immune cells and their ability to synthesize locally C1q (and/or other complement components), and most crucially, the biochemical nature of the tumor microenvironment.

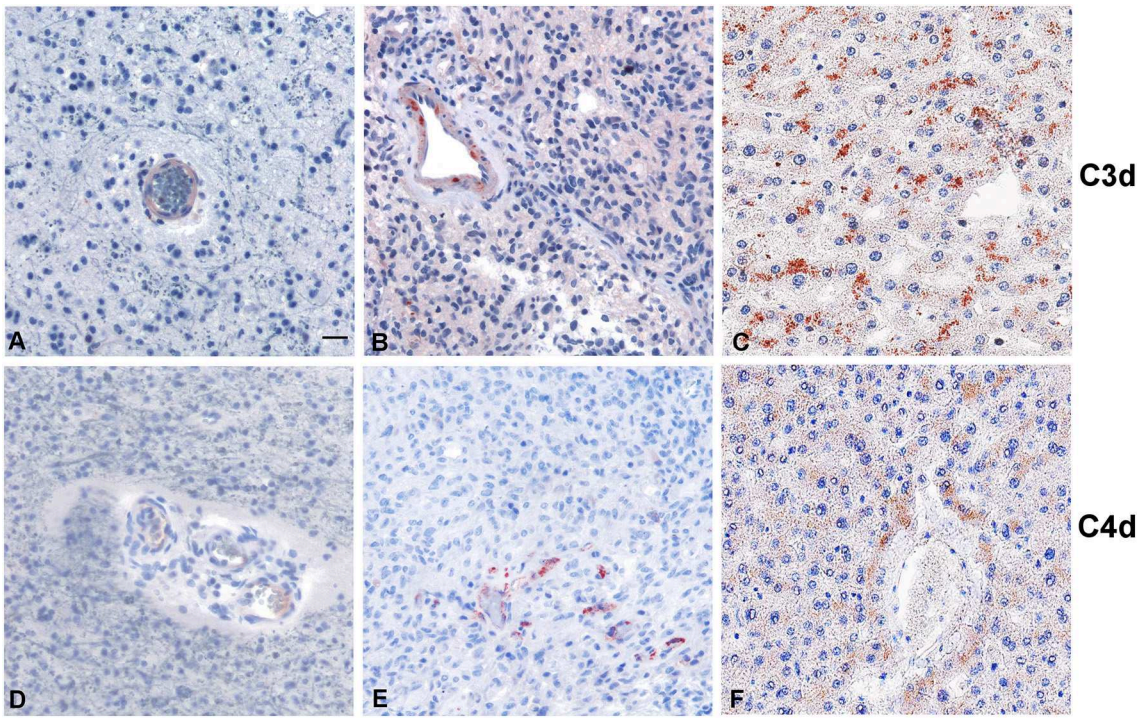

FIGURE 5 | Representative immunohistochemical analysis of C3d and C4d complement activation products in low grade **(A,D)** and high grade **(B,E)** gliomas. C3d and C4d specific staining is segregated in the blood vessels, while the tumor tissues are negative. Liver staining **(C,F)** represents the antigen (tissue) control. Polymer detection system with AEC (red) chromogen; scale bars, 50 μm.

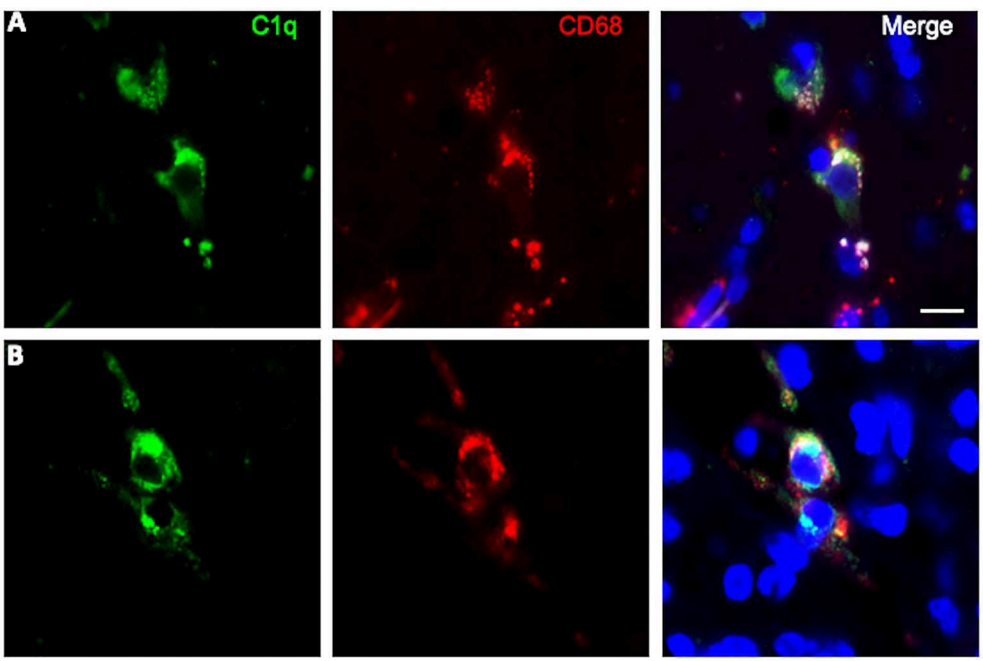

FIGURE 6 | Representative microphotographs of double immunofluorescence for C1q (green signal) and CD68 (red signal) in FFPE sections of low **(A)** and high **(B)** grade gliomas confirming the macrophage nature of C1q expressing monocytoid elements. The cell nuclei were stained with DAPI; scale bars, 10 μm.

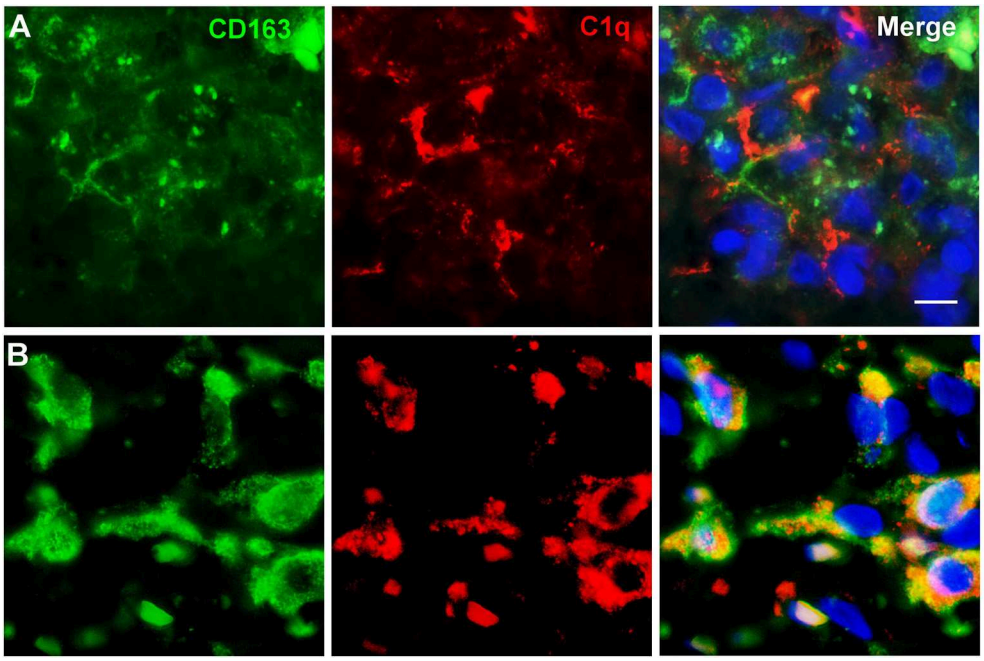

FIGURE 7 | Representative microphotographs of double immunofluorescence for C1q (red signal) and CD163 (green signal) in FFPE sections of low **(A)** and high **(B)** grade gliomas confirming the M2-polarization of macrophages expressing C1q. The cell nuclei were stained with DAPI; scale bars, 10 μm.

In the brain, complement components, including C1q, can be locally produced by resident neurons and glial cells, microglia and astrocytes being the major producers (19, 36). Our immunocytochemical data demonstrated that CD68 and CD163 positive infiltrating cells represent the cell types actively synthesizing C1q in the tumor microenvironment (**Figures 6**, **7**). CD68 expression is characteristic of tumor-associated macrophages, whose enrichment in glioma has been associated with poor prognosis (37). CD163 identify the M2-polarized macrophages, which are highly versatile cells known to influence multiple steps in tumor development and invasiveness along with angiogenesis and immunosuppression (38).

These cells in the brain are derived from two different sources: resident microglia and monocytes/macrophages that enter the brain from bone marrow. Even though it is quite difficult to distinguish between these two different cell types due to the lack of available definitive markers, they are recruited by the tumor microenvironment via several gliomas derived chemokines that also contribute to their polarization from a tumor-suppressive to a tumor-promoting phenotype (39). It is interesting to note that C1q has been shown to enhance the secretion and action of these chemokines (40, 41). Therefore, C1q produced and released by microglia/macrophage cells, is expected to promote immunosuppression, thus favoring glioma cell proliferation. This is consistent with our observation linking the expression of the three chains of human C1q with an unfavorable prognosis in grade-II and -III gliomas (LGGs) in TCGA dataset and in grade-III gliomas in CGGA dataset, respectively (**Figures 2**, **3**).

We also observed a high degree of deposition of C1q in the perivascular stroma as well as on endothelial cells belonging to the tumor vasculature. Developing gliomas require an increased nutrient supply and hence trigger neovascularization via the release of angiogenetic factors by cancer cells. C1q itself promotes angiogenesis through its globular heads (7). Massive angiogenesis is induced by regions of hypoxia within the tumor that are not only loci generating damage-associated molecular patterns (DAMPs), recognized by C1q but also privileged sites for glioma stem-like cell (GSC) settlement (42). These cells are quite dangerous since they maintain tumor growth through self-renewal amidst a supportive microenvironment. C1q, in this context, has been shown to participate in GSC maintenance and expansion via the activation of the canonical Wnt signaling cascade through its binding to Frizzled-receptor (43).

Our bioinformatics study, based solely on mRNA expression dataset and therefore further requiring a validation at the protein level, underlines how complex, multifaceted and yet feebly understood is the differential role of C1q in tumor progression or suppression. In conclusion, C1q plays a fundamental role in the pathogenesis of gliomas, but further investigation is required for its use in the clinic as a prognostic marker.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: TCGA-GBM, TCGA-LGG (https://tcga-data.nci.nih.gov/tcga/), and CGGA (http://www.cgga.org.cn).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of government guidelines, and approved by the CEUR (Comitato Etico Unico Regionale, FVG, Italy; number 34/2016). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AMan, PZ, and RB: conceptualization and writing–original draft preparation. AMar and BB: methodology. AMan: software and visualization. DB: validation. FZ: formal analysis. BB and DB: investigation. AMan, VR, DI, and FZ: resources. CA: data curation. PZ, CA, and UK: writing–review and editing. RB: supervision and project administration.

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