Thesis

Functional consequences of anti-C1q autoantibodies from systemic lupus erythematosus patients

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For my parents,

for their endless love and support.

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Abbreviations

AB	antibody
AD	assay diluent
Ag(s)	antigen(s)
ANA	anti-nuclear antibodies
anti-C1q	anti-C1q autoantibodies
anti-dsDNA	anti-double stranded DNA autoantibodies
AnV	AnnexinV
AP-	alkaline phosphatase
AP	alternative pathway
APC	Allophycocyanin
APCs	antigen-presenting cells
ASA	Acetylsalicylacid
AU	arbitrary units
AZA	Azathioprine
biot.	biotinylated
biot-C1q	biotinylated C1q
BSA	bovine serum albumin
$C1q^{-/-}$ mice	C1q-deficient mice
C1qDS	C1q-depleted serum
C1q-ICs	C1q-bearing immune complexes
C1qR(s)	C1q receptor(s)
Ca^{2+}	Calcium
cC1q	collagen-like region of C1q
CD	cluster of differentiation
CLIP	class II-associated invariant chain peptide
CLR	collagen-like region of C1q
CP	classical pathway
CR1/2	complement receptor ½
CRP	C-reactive protein
CRT	Calreticulin
CTX	Cyclophosphamide
DAF	decay accelerating factor
DCs	dendritic cells
denat. C1q	denatured C1q
Dexa	Dexamethasone
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinases
f	female
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FcγR(s)	Fcgamma receptor(s)
FITC	Fluorescein isothiocyanate
gC1q	globular head region of C1q
GM-CSF	granulocyte macrophage-colony stimulation factor
gMFI	geometric mean fluorescence intensity
HCQ	Hydroxychloroquine
HMDMs	human monocyte-derived macrophages
HRP	horseradish peroxidase
HSA	human serum albumin
HS-PBS	high-salt PBS
HUVS	hypocomplementemic urticarial vasulitis syndrome
iC1q	intracellular C1q
ICAM	intracellular adhesion molecule
ICs	immune complexes
iDCs	immature dendritic cells
IFNγ	Interferon gamma
Ig	immunoglobulins
Ig-class	immunoglobulin-class
Ii	invariant chain
IL-	interleukine-
IL-1RA	IL-1 receptor antagonist
imC1q	immobilized C1q
imC1q+NHIgG	NHIgG incubated on imC1q
imC1q+SLEIgG	anti-C1q bound to imC1q
int	intermediate
IQM	interquartile mean
JAK	Janus kinases
KCs	Kupffer cells
kDa	kilo Dalton
LAIR-1	leukocyte-associated Ig-like receptor 1
LDL	lipoproteins
LMW-C1q	low-molecular-weight C1q
LP	lectin pathway
LPS	lipopolysaccharide
LTA	lipoteichoic acid
m	male
M1	proinflammatory macrophages
M2	anti-inflammatory macrophages
MAC	membrane attack complex

MAC-IP	MAC-inhibitory protein
MAPK	mitogen-activated protein kinase
MBL	mannose-binding lectin
mC1q	membrane-bound C1q
MCP	membrane cofactor protein
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage-colony stimulating factor
mDCs	mature dendritic cells
MerTK	Mer tyrosine kinase
MFI	mean fluorescence intensity
Mg^{2+}	Magnesium
MHC class I/II	major histocompatibility complex class I/II
MLR	mixed lymphocyte reaction
MMF	Mycophenolate mofetile
MTX	Methotrexate
na	not available
NF-κB	nuclear factor-κB
NHIgG	normal human IgG
NHS	normal human sera
LN	lupus nephritis
ns	not significant
NSAIDs	non-steroidal anti-inflammatory drugs
nt	not tested
pat	patient
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pDCs	plasmacytoid DCs
PD-L	programmed cell death protein-1
PD-L1/2	programmed cell death ligand-1/2
PE	Phycoerythrin
PGN	peptidoglycan
PI	Propidium iodide
PMA	phorbol 12-myristate
pMac	peritoneal macrophages
Pred	Prednisone
PS	phosphatidylserine
PTX3	pentraxin 3
R	Spearman's rank correlation coefficient
RA	rheumatoid arthritis
Ref	reference
RIT	Rituximab
RPMI	Roswell park memorial institute (media)
RT	room temperature
sC1q	soluble C1q
SIV	simian immunodeficiency virus
SLE	systemic lupus erythematosus
SLEIgG	SLE patient-derived IgG
SN	supernatants
SRBCs	sheep red blood cells
STAT	signal transducers and activators of transcription
TAMs	tumor-associated macrophages
TGF-β	tissue growth factor-beta
TLR(s)	toll-like receptor(s)
TNFα	tumor necrosis factor alpha
VBS	veronal buffered saline
y	years

General summary and aim of the thesis

The complement system is a key component of the innate immune system (1). It consists of several plasma and cell-associated proteins and acts as an enzymatic-driven protein cascade. The complement system can be activated via at least 3 different pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP) (1, 2). C1q, the recognition and starter molecule of the CP of complement, is considered to be involved in the pathogenesis of systemic lupus erythematosus (SLE). This observation is based on the fact that homozygous C1q deficiency is the strongest known genetic disease susceptibility for the development of SLE in humans (3, 4). In addition, C1q-deficient mice develop a SLE-like disease (5). However, most SLE patients do not suffer from primary C1q deficiency, moreover, aberrant complement activation is accounted for secondary hypocomplementemia (6). Another reason for low C1q levels are autoantibodies directed against C1q (anti-C1q) which are present in 20-50% of SLE patients. The occurrence of anti-C1q not only correlates with hypocomplementemia, but additionally, also with the occurrence of severe lupus nephritis (7-9). The association of anti-C1q with lupus nephritis suggests a pathogenic role of these autoantibodies in this inflammatory kidney disease. Even though, anti-C1q correlate with renal involvement, the direct evidence how these autoantibodies contribute to the pathogenesis of lupus nephritis is not yet available. Animal studies demonstrated that anti-C1q were only pathogenic in combination with predeposited glomerular C1q-containing immune complexes. Trouw et al. proposed that extensive anti-C1q-mediated complement activation might damage the kidney and lead to the infiltration of immune cells resulting in glomerular injury by Fcgamma receptor (FcγR)-mediated mechanisms suggesting a role for both, the complement system as well as phagocytes, in lupus nephritis (10). Nevertheless, little knowledge is available on the pathogenic properties of these autoantibodies and their biological function is not well defined.

The aim of my thesis was to analyze the interaction of anti-C1q with the complement system and the down-stream effects of anti-C1q on macrophages. The following questions have been addressed during my thesis:

Part 1: Do anti-C1q trigger the activation of the complement system?

So far, it was not known if anti-C1q influence the activation of the complement system. In nephritic kidneys of SLE patients co-localization of complement components (C1q, MBL, C4, C3) and immunoglobulins (Ig; IgG, IgA, IgM) occurs (11, 12), suggesting that the complement system is involved in the pathogenesis of lupus nephritis (13). Anti-C1q can be of the IgG-, IgA-, and IgM-class (14-16). The anti-C1q Ig-class repertoire might indicate that anti-C1q bound to immobilized C1q (imC1q) could activate the complement system via its different pathways. Indeed, when evaluating the relative contribution of each complement pathway in the complement-activating potential of anti-C1q using an ELISA-based assay, we found that SLE patient-derived anti-C1q amplify complement activation *in vitro* via the CP and the LP dependent on the anti-C1q Ig-class repertoire present in the lupus patient's serum with IgG anti-C1q primarily activating the CP, IgA the LP, and IgM both the CP and the LP. In addition, bound anti-C1q resulted in the activation of the CP as reflected by C4b deposition in the presence of purified C1 and C4 in a dose-dependent manner. The extent of C4b deposition correlated with IgG anti-C1q levels of SLE patients but not of healthy controls. These findings are of importance for the understanding of the role of anti-C1q in SLE suggesting a direct link to hypocomplementemia.

Part 2: Do anti-C1q induce a proinflammatory phenotype in macrophages?

Functional defects in myeloid cells obtained from SLE patients are well known, although the underlying molecular mechanism is not fully understood. Macrophages and monocytes obtained from SLE patients exhibit a defect in the phagocytosis of apoptotic cell material (17-19), which contributes to the accumulation of a large number of apoptotic cells in various tissues (20, 21). Furthermore, C1q facilitates the uptake of apoptotic cell material by monocytes, macrophages, and dendritic cells (DCs) (22-24). During the phagocytosis of apoptotic cells, C1q exerted a potent inhibitory capacity in macrophage-mediated inflammation (25). These data suggest that C1q is crucial in limiting inflammation during the uptake of apoptotic cells. In this context, it has been hypothesized that the binding of anti-C1q to C1q might interfere in the immune-regulatory functions of this molecule. However, the direct downstream effect of anti-C1q on professional phagocytes is not well understood. Therefore, we developed an *in vitro* model to study the effect of SLE patient-derived anti-C1q bound to imC1q on human monocyte-derived macrophages (HMDMs) obtained from healthy donors and SLE patients. Morphologically, bound anti-C1q induced the formation of cell aggregates of HMDMs when compared to imC1q or IgG alone. In addition, anti-C1q reversed the effect of

imC1q alone shifting the lipopolysaccharide (LPS)-induced cytokine release towards a proinflammatory response. By FcγR-blocking experiments, the secretion of proinflammatory cytokines was found to be mediated via FcγRII. The anti-C1q-induced inflammatory cytokine profile was accompanied by a downregulation of CD163 and an upregulation of LPS-induced CD80, CD274, and MHC class II. Finally, HMDMs primed on bound anti-C1q versus imC1q alone displayed a significantly lower phagocytosis rate of early and late apoptotic cells accompanied by a reduced Mer tyrosine kinase expression. Interestingly, anti-C1q-dependent secretion of proinflammatory cytokines was similar in SLE patient-derived cells with the exception of IL-10 being slightly increased. In conclusion, anti-C1q induced a proinflammatory phenotype in HMDMs reversing the effects of imC1q alone. This effect might exacerbate underlying pathogenic mechanisms in lupus nephritis.

Part 3: Do anti-C1q influence C1q secretion by macrophages?

As opposed to most other complement proteins, C1q has a non-hepatic origin and is predominantly produced by myeloid cells, i.e., DCs and macrophages (26). In tissues, C1q is accumulated during inflammatory processes due to local production by infiltrating DCs and macrophages (27, 28). In the glomeruli of patients with proliferative nephritis, deposited C1q as well as anti-C1q can be found (11, 12). In addition, anti-C1q strongly correlate with the occurrence of lupus nephritis (7-10). In parallel, anti-C1q seem to influence C1q levels, as anti-C1q negatively correlate with C1q levels (29, 30). In this context, anti-C1q might be able to directly alter C1q secretion by HMDMs. Therefore, we investigated the C1q secretion profile of HMDMs obtained from healthy donors and SLE patients *in vitro*. In contrast to our expectations, we observed that bound anti-C1q induced significantly higher C1q secretion levels as compared to imC1q alone or healthy donor IgG. The extent of C1q secretion by HMDMs correlated with IgG anti-C1q levels of SLE patients but not of healthy controls. Furthermore, bound autoantibodies and imC1q induced continuous and *de novo* C1q synthesis as evident by the stored intracellular C1q content which correlated with C1q secretion levels. In addition, secreted C1q was able to activate the CP as reflected by C4b deposition in a dose-dependent manner. Interestingly, anti-C1q-dependent C1q secretion was similar in SLE patient-derived cells. In conclusion, our data indicate that imC1q-bound anti-C1q strongly stimulate C1q production by HMDMs. Thus, bound anti-C1q can induce a potent C1q-producing phenotype in macrophages. This enhanced C1q secretion might potentiate anti-C1q induced complement activation and the binding of additional anti-C1q molecules leading to a local vicious circle.

Taken together, I can demonstrate that anti-C1q activate the complement system via the CP and the LP but not via the AP dependent of the anti-C1q Ig-class present in the patient's sera (see part 1, p. 29). Furthermore, I show that anti-C1q induce a proinflammatory phenotype in HMDMs via an FcγRII-dependent pathway (see part 2, p. 46). In addition, I can also demonstrate that anti-C1q trigger C1q secretion by HMDMs (see part 3, p. 73). Moreover, I can show that the inhibition of Cathepsin S can suppress the anti-C1q-triggered induction of a proinflammatory phenotype in HMDMs (data not shown).

The thesis is divided into a general introduction summarizing the current knowledge on topics relevant for this thesis followed by 3 main parts summarizing manuscripts. The results of the first study (part 1) are published in *Clinical Immunology*. The manuscript of part 2 is currently under revision and the manuscript of part 3 was recently submitted for publication.
The thesis is completed by my conclusions and an outlook of ongoing and future projects.

General introduction

The complement system

The complement system is part of the innate immune system. Its biological functions include host defense by opsonization of invading pathogens followed by complement-mediated cell lysis, clearance of immune complexes (ICs) and apoptotic cell material, as well as bridging adaptive with innate immunity (1, 31). The complement system consists of several plasma and cell-associated proteins, which either participate in the complement cascade itself or are regulators of complement activation. The complement system acts as an enzymatic protein cascade and its activation can occur via at least 3 different pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP) followed by a terminal pathway common to all (Figure 1). Each of these pathways has its own activating recognition mechanism, i.e., is activated by its own specific ligand (1, 2).

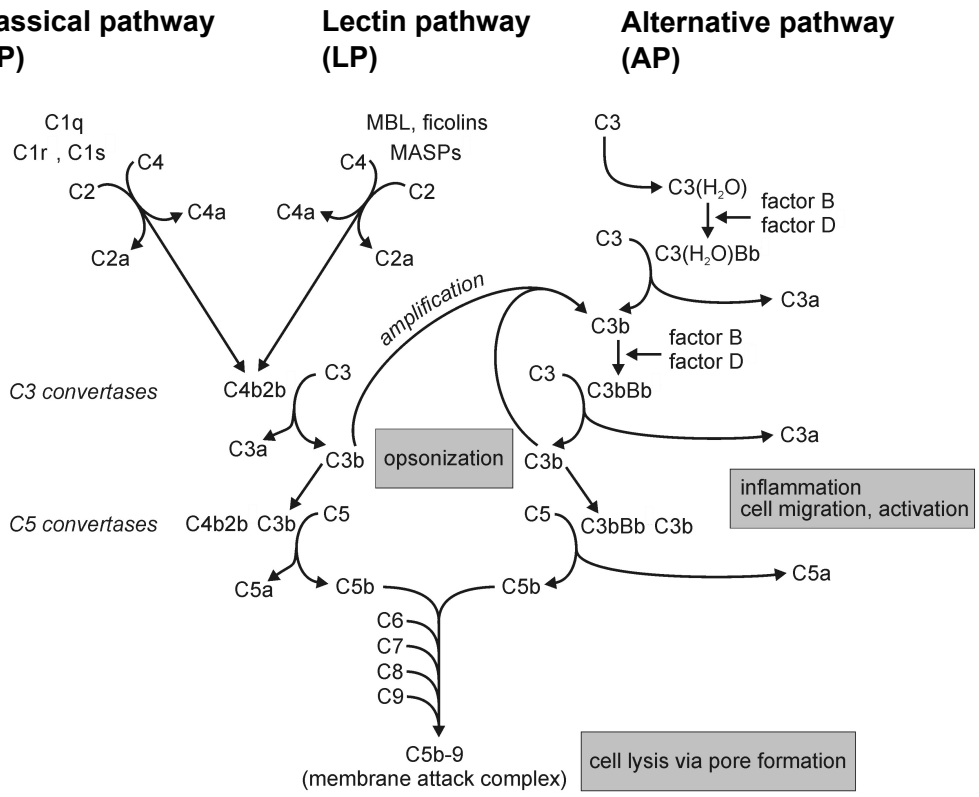


Figure 1: Overview of the complement system.

The complement system can be activated via the CP, the LP, or the AP depending on the activating ligand. Upon activation, all 3 pathways converge at the level of the C3 convertase. Downstream activation of terminal complement proteins (C5 to C9) leads to the formation of the membrane-attack complex which is inserted into the target cell wall causing cell lysis (modified according to (32)).

The CP (Figure 2) is activated upon binding of C1q, the recognition molecule of C1 (a complex consisting of C1q and 2 serine proteases C1r and C1s), to Fc regions of immunoglobulins (Ig; IgM- and certain IgG-classes (IgG1, IgG2, and IgG3 in humans)) which are bound to antigens (Ags). Upon binding of C1q to its ligands, C1q changes its conformation resulting in subsequent activation of C1r which then activates C1s. C1s further cleaves C4 into its 2 fragments, C4a and C4b. The latter is deposited onto the cell membrane. Further activation of the complement cascade leads to the generation of the CP-C3 convertase (C4b2a) (1, 33).

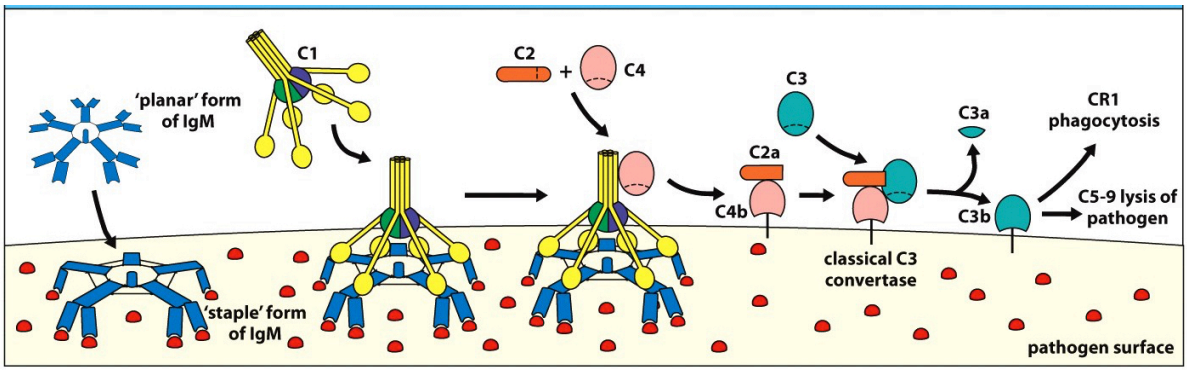


Figure 2: Activation of the CP by IgM binding to Ags on the surface of a pathogen.

The CP is triggered by an activated C1 complex which is composed of the recognition molecule C1q and its 2 serine proteases C1r and C1s. Binding of C1q to the Fc region of certain Ig (IgM and certain IgG-classes) in ICs leads to the activation of the C1 complex. Upon binding of C1q to a ligand, C1q activates C1r which in turn activates C1s that cleaves C4 into C4a and C4b. C4b binds covalently to the surface of a pathogen. Then, C4b binds C2 which is cleaved by C1s into C2a and C2b. C2a forms together with C4b the CP-C3 convertase (C4b2a). Downstream activation of terminal complement proteins (C5 to C9) leads to the formation of the MAC which is inserted into the cell wall of the pathogen causing its lysis (obtained from (34)).

The AP is initiated by spontaneous C3(H_2O) binding and activation/turn-over, or by direct binding to microbial structures (e.g., lipopolysaccharide (LPS)) leading to the cleavage of C3 into C3a and C3b or as an amplification loop by C3b deposition on cell surfaces after initial activation of the CP or the LP (1, 33).

The recently described LP is usually not activated by antibodies but by binding of mannose-binding lectin (MBL) or ficolins to, e.g., mannose residues exposed on the surface of certain pathogens (1, 33).

Independent of the pathway which has been initially activated, all 3 pathways converge in the formation of the C3 convertase which cleaves C3 into C3a and C3b. Finally, downstream activation of terminal complement components (C5 to C9) leads to the assembly of the C5b-

C9 complex (termed membrane-attack complex (MAC)) which is inserted into the target cell wall causing cell lysis (33, 35).

During the activation of the complement system, complement fragments are generated by cleavage, such as anaphylatoxins (namely C3a and C5a) as well as opsonins (inactivated C3b (iC3b)) (36). Anaphylatoxins act as potent chemotactants for several immune cells, such as neutrophils, mast cells, or macrophages, and take part in inflammatory processes (37). Additionally, many cell types express receptors for intact or cleaved complement proteins, e.g., complement receptor 1 (CR1, CD35) and complement receptor 2 (CR2, CD21) or receptors for C1q (C1qRs) (data not shown) (38).

Regulation of the complement system

To prevent self from complement-mediated damage, the complement system is tightly regulated (Figure 1). For this purpose, most mammalian cells express regulatory proteins on their surface to control complement activation, e.g., CR1, membrane cofactor protein (MCP, CD46), decay accelerating factor (DAF, CD55), and MAC-inhibitory protein (MAC-IP, CD59). Additionally, the complement system is regulated by several plasma proteins at different stages of the complement cascade, e.g., C1-inhibitor, C4b-binding protein, factor I, factor H, and Properidin (38, 39).

Systemic lupus erythematosus

In Northern Europe, about 40/100'000 people are affected by this incurable chronic autoimmune disease. However, the incidence varies between geographic regions, ethnical groups, genders, and age. Systemic lupus erythematosus (SLE) is most common in women during their childbearing years and in black Americans as well as Hispanics (40, 41). It is characterized by a wide range of clinical and immunological manifestations and the potential involvement of multiple organs, including joints, central nervous system, skin, heart, blood vessel, and kidneys, leading to a variety of symptoms. The disease course often follows phases of relapses and remissions (42). The etiology of this autoimmune disease is mostly unknown and seems to involve genetical, hormonal, and environmental factors (43).

SLE is characterized by B cell hyperactivity, antibodies specific for various auto-Ags such as intra-cellular components (e.g., anti-double-stranded DNA autoantibodies (anti-dsDNA)) or plasma proteins (e.g., anti-C1q autoantibodies (anti-C1q)), the formation of ICs, and the aberrant activation of the complement system resulting in complement deposition and hypocomplementemia (44, 45).

The complement system and SLE

The complement system has an important role in the pathogenesis of SLE. In particular, deficiencies of the early complement proteins of the CP (C1q, C4, C2) have been associated with an increased risk to develop SLE (3). Nevertheless, the highest known susceptibility in humans to develop SLE has been described in individuals suffering from homozygous C1q deficiency (46, 47). However, most SLE patients do not suffer from primary deficiency in complement proteins (3). In general, low circulating complement levels are considered to reflect aberrant complement activation, i.e., so-called secondary hypocomplementemia.

The clearance of apoptotic cell material and SLE

Furthermore, the strong link between C1q deficiency, the development of SLE, and the clearance of apoptotic cells led to the formulation of the so-called *"waste disposal hypothesis"*: Next to its involvement in host defense, the complement system has multiple other functions, including the clearance of apoptotic cell material (1). The efficient and fast clearance of early apoptotic cell material is crucial to avoid inflammatory and autoimmune processes (48-50). More importantly, dying cells and inefficiently cleared apoptotic cells might serve as an accessible source of intracellular Ags (such as dsDNA) and trigger autoantibody production in susceptible individuals (51, 52). Experiments in mice support this hypothesis. Mice having an inefficient removal of apoptotic cells develop severe autoimmunity characterized by the

occurrence of autoantibodies directed against nuclear components and resembling human SLE (53). Vice versa, lupus-prone mice have an impaired clearance of apoptotic cell material (54). Injection of an excess of apoptotic thymocytes into healthy mice induced the production of autoantibodies, including anti-nuclear antibodies (ANA) and anti-dsDNA (55). Therefore, a defective clearance of apoptotic material by macrophages has been proposed as the pathogenic mechanism underlying SLE, causing an accumulation of dead cell material resulting in inflammation and autoimmunity (20, 50). Indeed, it is well established that SLE patient-derived macrophages exhibit a defect in the clearance of apoptotic cell material correlating with low complement levels (17, 18). Moreover, many targets for lupus patient-derived autoantibodies are concentrated on the surface of apoptotic cells (56).

Based on the observations reported above, a defective clearance of apoptotic cells has been suggested to break peripheral tolerance, provide a reservoir of self-Ags inducing autoantibody production, and finally lead to the development of SLE (57-60).

The complement protein C1q

C1q (Figure 3) is a 460kDa collagen-like, hexametric glycoprotein which belongs to the family of the so-called collectins. The intact C1q molecule consists of 3 distinct polypeptide chains (C1qA, C1qB, and C1qC chains). Each of these chains occurs 6 times in the protein adding up to a total of 18 chains. The 3 different polypeptide chains are translated from 3 distinct C1q genes (C1qA/B/C genes), which are all located on chromosome 1. Furthermore, each chain consists of an amino-terminal collagen-like region (cC1q) and a carboxyl-rich terminal globular head region (gC1q). One C1qA and one C1qB chains are covalently linked together to form an A-B dimer and 2 C1qC chains to form a C-C dimer, respectively. Additionally, one A-B dimer is non-covalently linked to one C1qC chain of a C-C dimer forming one triple helical strand. In total, 6 triple helical strands form the C1q molecule. A doublet is then assembled by 2 of these strands through a disulfide bond of 2 C1qC chains. 3 of these doublets build up the intact C1q molecule that due to its organization resembles a bouquet of tulips (61-64).

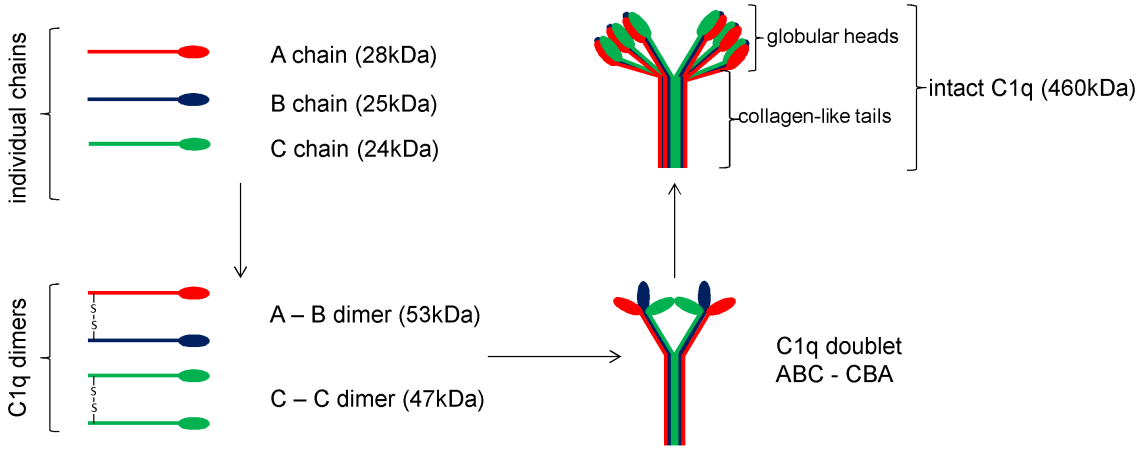


Figure 3: Overview of the structural organization and subunit assembly of intact C1q.

One C1qA chain is covalently linked to one C1qB chain through a disulfide bond (A-B dimer). A triple helix is formed by an A-B dimer non-covalently linked to a C1qC chain of a C-C dimer. 6 of these triple helices are formed by the 18 polypeptide chains forming the intact C1q molecule. Each chain consists of a collagen-like region and a globular head region (modified according to (64)).

In the blood circulation, C1q is mostly associated with its 2 serine proteases $C1s_2$-$C1r_2$, in a calcium-dependent manner, to form the C1 complex, the starter molecule of the CP (61, 63, 65, 66). The concentration of C1q (complexed) in sera of healthy individuals varies from 80-180µg/ml (67). However, under physiological conditions in healthy individuals the occurrence of free C1q is mostly limited to tissues due to local C1q production by myeloid cells, i.e.,

macrophages and dendritic cells (DCs) (26, 68-70). The secretion and functions of C1q in immune cell modulation will be discussed later (see p. 12/16).

Functions of C1q

Beyond its function as the recognition molecule in the C1 complex, initiating the activation of the CP upon binding to ICs, C1q has many other functions (Figure 4).

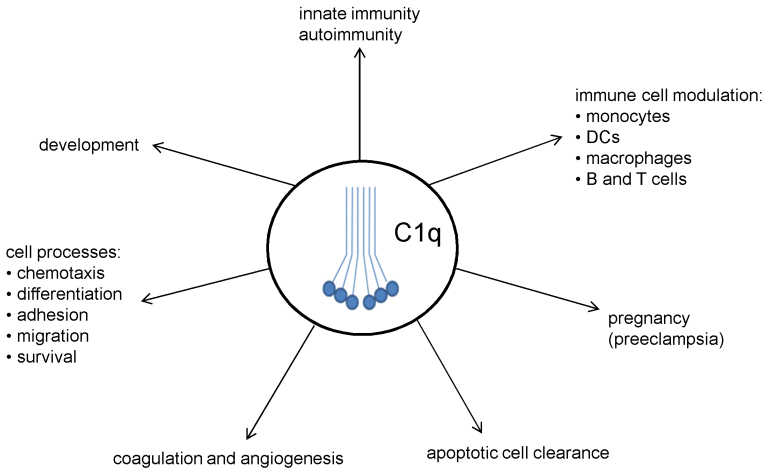


Figure 4: Classical and non-classical functions of C1q.

C1q has many other functions in the human body which are not directly related to complement activation. Some are listed in the figure (modified according to (71)).

C1q is involved in many pathological conditions, including autoimmune diseases (e.g., SLE), cancer, atherosclerosis, and preeclampsia (72-75).

For example, C1q is involved in the clearance of apoptotic cell material. It has been demonstrated that C1q binds to the surface of apoptotic cells and facilitates their uptake by phagocytes (22, 76-78). As outlined before, C1q-deficient ($C1q^{-/-}$) mice develop an autoimmune phenotype resembling human lupus characterized by an accumulation of apoptotic cells in the kidneys (5), underlining the importance of C1q in the clearance of apoptotic cell material.

Furthermore, C1q serves as a pattern recognition molecule binding to numerous ligands, including DNA, annexins A2 and A5, phosphatidylserine (PS), and LPS (79-81), thereby recognizing pathogens and apoptotic cells and modulating biological and cellular responses (reviewed in (82, 83)). C1q also regulates cell differentiation, adhesion, chemotaxis, migration, activation, and survival (84-87). C1q's function in immune cell modulation will be discussed later (see p. 16).

C1q production and secretion

Most complement proteins have a hepatic-origin and are produced and regulated as acute phase proteins. Nevertheless, many complement components have next to the liver also other production sites (88, 89). Indeed, many immune cells are able to produce functional complement proteins. For example, several studies reported that DCs and macrophages produce a variety of different complement proteins, including C1s, C4, C2, C3, C5, and C9 (68, 90-92). This synthesis is differently regulated in response to inflammatory stimuli, such as LPS or cytokines (26, 90).

As opposed to most complement proteins, C1q has mainly a non-hepatic origin (88). Studies of liver-derived cells, isolated from guinea pigs (93) and rats (mainly Kupffer cells (KCs)) (94), confirmed that C1q may also have a hepatic-origin, however, C1q mRNA levels in liver-derived cells from guinea pigs were at considerably lower expression levels as compared to those of recruited peritoneal macrophages (pMac) (93). In addition, it has been reported that in mouse organs (liver, lung, and small intestine) only trace amounts of C1q-specific mRNA can be detected (95), suggesting that liver cells are not the main source of serum C1q. So far, many different cell types have been described to produce and secrete C1q, including epithelial cells and fibroblasts (96, 97). An overview of cell types producing C1q is listed in Table 1.

Table 1: Overview of C1q-producing cells in humans, guinea pigs, mice, and rats.

Species	C1q-producing cell type	Ref
human	monocytes	(98, 99)
	→ no C1q secretion but positive for C1q mRNA (donor-specific)	
	DCs: iDCs>>mDCs	(100-102)
	→ independent of the maturation stimuli used (LPS, IFNα, …)	
	macrophages (HMDMs, pMac)	(69, 70, 103, 104)
	osteoclasts	(105)
	fibroblasts	(96, 106-108)
	endothelial cells	(109)
	epithelial cells	(97)
	mesenchymal cells	(97)
	microglial cells	(110, 111)
	trophoblasts	(109, 112)
guinea pig	pMac	(103)
	hepatocytes (hepatocyte primary cell culture)	(93)
mouse	bone marrow stromal cells (macrophages, DCs)	(113)
	macrophages	(114)
	pMac	(95)
rat	C1q-expressing spleen cells (macrophages, DCs)	(115)
	liver-derived mononuclear phagocytes (KCs)	(94)

Legend: HMDMs: human monocyte-derived macrophages; iDCs: immature dendritic cells; mDCs: mature dendritic cells

Nevertheless, it is believed that C1q is predominantly produced by myeloid cells (26, 68-70). This hypothesis is based on the fact that $C1q^{-/-}$ mice, lacking detectable serum C1q levels, were able to restore normal C1q serum levels upon receiving a bone marrow transplant from wild type mice, and vice versa (116). Interestingly, monocytes fail to synthesize C1q, even though, some cells have been tested positive for C1q mRNA (98, 99). However, monocytes gain the ability to produce C1q during their differentiation into macrophages or DCs (26, 68-70). C1q is expressed by bone marrow stromal DCs and macrophages (113). In particular, iDCs are an important source of functional C1q, but upon maturation they downregulate this capability (26, 100). Besides DCs, macrophages as well produce C1q, although their secreted C1q levels are lower as compared to those of DCs (unpublished data; (26)). Interestingly, subsets of macrophages (see p. 25/unpublished data) differ in their C1q producing capacity. Even though, resident macrophages produce constantly low C1q levels, their proinflammatory counterparts are able to produce much higher C1q levels (117, 118). It is hypothesized that the modulation of C1q secretion by macrophages in response to different stimuli is dependent on the developmental stage of these cells. HMDMs kept in culture for several weeks constantly secreted C1q (69) and secreted C1q was found to share similarities to serum C1q (68, 70). In addition, C1q secreted by iDCs and macrophages was functionally active in the activation of the CP and in binding to apoptotic cells (S. Thanei et al. (submitted); see part 3, p. 73, (26, 70)). Therefore, C1q secreted and produced by immune cells is considered to have an important role in the local environment of immune cells, at sites of inflammation where macrophages and DCs are present and freshly synthesized C1q might accumulate.

In vitro, C1q synthesis and expression can be influenced by different agents, including LPS, steroids, cytokines (IFN-γ, IL-6), C3b-opsonized zymosan, and ICs (27, 69, 104, 119). Notably, immobilized C1q (imC1q) was shown to trigger its own production in DCs but not in HMDMs (120). In contrast, Galvan et al. reported that imC1q also upregulates C1q mRNA in murine macrophages (121). These reports suggest that imC1q itself can lead to an elevated C1q production consistent with our findings (S. Thanei et al. (submitted); see part 3, p. 73). Studies addressing how C1q secretion and production can be influenced in different cell types by different agents are summarized in Table 2.

Table 2: Regulation of C1q expression and secretion in different cell types.

Only papers analyzing isolated mononuclear cells or cell lines have been included.
C1q expression: Refers to the expression of C1q mRNA
C1q secretion: Refers to the secretion of C1q into supernatants (SN) of cell cultures

Table 2.1: Upregulation of C1q expression and secretion.

Stimulatory agent used	Mechanism of regulation C1q-producing cells/species	Ref
TLR ligands (LPS, LTA, PGN)	- Induction of C1q secretion by human iDCs	(122)
LPS	- Upregulation of C1q expression and secretion by human and murine macrophages - Induction of C1q expression by THP-1 cells	(26-28, 123)
IL-6	- Upregulation of C1q expression and secretion by murine pMac and THP-1 cells	(119, 123)
IFNα/β	- Upregulation of C1q expression and secretion by murine macrophages	(28, 114)
IFNγ	- Induction of C1q production by THP-1 cells and murine microglia cells - Induction of C1q expression and secretion by murine macrophages	(27, 28, 114, 123-125)
Steroids (Hydrocortisone, Pred, Dexa)	- Upregulation of C1q gene expression and/or secretion by KCs and human pMac - Induction of C1q production but not C1q secretion by murine (resident) pMac - Stimulation of C1q production by THP-1 cells	(94, 104, 118, 123)
NSAIDs	- Induction of C1q production but not C1q secretion by murine (resident) pMac	(118, 119)
imC1q	- Upregulation of C1q mRNA expression by murine macrophages - Induction of C1q secretion by HMDMs (study dependent) and DCs	(120, 121) (S. Thanei (sub.))
C3b-opsonized zymosan	- Induction of C1q expression and secretion by murine macrophages upon C3bR triggering	(27)
anti-C1q bound to imC1q	- Induction of C1q secretion and *de novo* synthesis by HMDMs	(S. Thanei (sub.))
ICs	- Induction of C1q expression and secretion by murine macrophages upon FcγR triggering	(27)
SIV	- Upregulation of C1q expression by rhesus macaques-derived microglia cells	(126)
tumor cells	- Upregulation C1q expression by murine TAMs	(127)

Table 2.2: Inhibition of C1q expression and secretion.

Inhibitory agent used	Mechanism of regulation C1q-producing cells/species	Ref
NSAIDs	- Inhibition of C1q production by murine pMac - ASA: decreased C1q secretion by human pMac	(104, 118)
IFN-γ	- Downregulation of C1q gene expression by rat-derived KCs and murine microglia cells - Suppression of C1q expression and secretion by murine pMac	(94, 119, 128, 129)
IL-1	- Inhibition of C1q expression by murine pMac	(119, 128)
PMA	- Suppression of C1q production by THP-1 cells	(123)
Tacrine	- Inhibition of C1q secretion in IFNγ-treated THP-1 cells by Tacrine, but not by Indomethacin, Cimetidine, or Propentofylline	(123)
LPS	- Downregulation of C1q gene expression by rat-derived KCs - Downregulation of C1q secretion during maturation process of human iDCs	(26, 94)
IFN-α	- Inhibition of C1q secretion by human iDCs	(26)

Table 2.3: No effect on C1q expression and secretion.

Stimulatory or inhibitory agent used	Mechanism of regulation C1q-producing cells/species	Ref
yeast	- No effect on C1q production by HMDMs	(69)
IgG-opsonized SRBCs	- No effect on C1q production by HMDMs	(69)

Legend: ASA: Acetylsalicylacid; Dexa: Dexamethasone; FcyR: Fcgamma receptor; LTA: lipoteichoic acid; NSAIDs: non-steroidal anti-inflammatory drugs; PGN: peptidoglycan; PMA: phorbol 12-myristate; Pred: Prednisone; SIV: simian immunodeficiency virus; SRBCs: sheep red blood cells; TAMs: tumor-associated macrophages

So far, the pathway and the mechanism leading to up- or downregulation of C1q synthesis and the regulation of the genes encoding C1q are not well understood. Locally secreted C1q probably acts in an auto-/paracrine manner maintaining tissue homeostasis by suppressing cell-mediated inflammation. Recently, we observed that HMDMs on imC1q and bound-anti-C1q continuously secreted high C1q levels and produced an accumulation of iC1q suggesting continued *de novo* synthesis. This observation indicates that high levels of secreted C1q are unlikely to act as a negative feedback mechanism thereby inhibiting the synthesis of fresh C1q (S. Thanei et al. (submitted); see part 3, p. 73). Furthermore, addition of exogenous C1q (20µg/ml) to cultured murine macrophages led to an increase in C1q mRNA levels (28). These reports suggest that C1q secretion might act as a positive feedback loop reflecting a temporal requirement of an increased amount of C1q in an inflamed tissue (130, 131). Local synthesis of C1q in tissues by myeloid cells is believed to play an important role in the clearance of

apoptotic cell material and in the recruitment of immune cells to sites of inflammation or injury *in situ*. Indeed, upregulated C1q production has been demonstrated in the brains of mice suffering from infections or Alzheimer's disease (130, 131), underlining the importance of local C1q production in the regulation of tissue homeostasis during inflammation. However, in the circulation of healthy individuals C1q is mostly associated with its proteases C1s and C1r to form the C1 complex (61, 63, 65, 66).

Notably, patients with active lupus having low serum C1q levels often display a serological abnormality: high levels of a non-functional form of C1q (so-called low-molecular-weight C1q (LMW-C1q)), which is unable to participate in the activation of the CP. This form of C1q was predominantly detected in SLE patients suffering from homozygous C1q deficiency (132). Nevertheless, LMW-C1q also occurred in SLE patients and healthy donors but not in rheumatoid arthritis (RA) patients or in patients suffering from acute poststreptococcal glomerulonephritis (132, 133). In addition, it has been demonstrated that SLE patient-derived macrophages produce elevated levels of LMW-C1q but the secretion of normal C1q was not impaired, indicating that LMW-C1q could be considered as a by-product of regular C1q synthesis (134). Furthermore, monocytes obtained from SLE patients without inherited C1q deficiency have a significantly impaired ability to upregulate C1q synthesis, both at the mRNA and protein levels, upon stimulation by Dexa and IFNγ, as compared to cells obtained from healthy donors or RA patients, underscoring the importance of C1q in autoimmunity and in particular in SLE (99).

C1q in immune cell modulation

The function of C1q is not only restricted to serve as the recognition molecule of the C1 complex and thus as the activator of the CP. The C1q molecule itself has an important function in immune cell modulation (summarized in Table 3).

As mentioned above, C1q serves as a regulatory protein during inflammatory processes including autoimmunity. Moreover, C1q participates in the clearance of apoptotic cell material. C1q can directly bind to the surface of apoptotic cells via its globular heads (76, 78). The collagen-like region (CLR) then interact with phagocytes via C1qRs (22) thereby facilitating the ingestion of apoptotic cells (23, 120). Additionally, studies from different research groups demonstrated that C1q not only enhances the uptake of apoptotic cell material but also modifies the cytokine profile released by phagocytic cells towards a less inflammatory response during phagocytosis, as evident by suppressed secretion of proinflammatory cytokines, including IL-1α/β, IL-6, and TNFα (23, 120, 135, 136). During the uptake of early and late apoptotic cells, C1q exerts a potent inhibitory capacity in macrophage-mediated

inflammation (24, 25). Furthermore, macrophages and DCs which ingested C1q bound to apoptotic cells significantly suppress the proliferation of Th17 and Th1 subsets (137). These data suggest that C1q is crucial in limiting inflammation during the uptake of apoptotic cells and proliferation of T cell subsets. Furthermore, C1q-polarization of macrophages induces an anti-inflammatory (M2-like) phenotype in macrophages (S. Thanei et al. (submitted); see part 2, p. 46).

Table 3: Summary of C1q- and bound anti-C1q-mediated effects on human immune cells.

Only papers focusing on isolated human-derived cells for *in vitro* experiments have been included.
imC1q: Refers to cells cultured on C1q immobilized/bound to a plastic surface
sC1q: Refers to cells incubated with fluid-phase C1q

Table 3.1: Papers analyzing the effect of sC1q.

Cell type	C1q-mediated effect	Ref
Monocytes	- Binding of sC1q to monocytes in a dose-dependent manner - Binding of sC1q to monocytes is partially mediated by LAIR-1: → binding of C1q triggers phosphorylation of LAIR-1 - Suppression of IFN type I production by CpG-challenged monocytes by C1q trough activating LAIR-1	(87, 138, 139)
HMDMs	- Binding of sC1q to HMDMs in a dose-dependent manner - Migration of M2-like HMDMs (IL-4) towards C1q → C1q induced migration: untreated macrophages<M1<M2a	(84, 138)
DCs	- Binding of sC1q to iDCs and mDCs in a dose-dependent manner - Chemotaxis of iDCs, but not of mDCs, in a dose-dependent manner → Migration mediated through ligation of both cC1qR and gC1qR - Differentiation of DCs in the presence of sC1q (tolerogenic DCs): → Enhanced phagocytic capacity → Impaired capacity to secrete cytokines (IL-6, TNFα, IL-10, IL-12p70) → Impaired upregulation of costimulatory molecules (CD80, CD83, CD86) upon LPS stimulation → Impaired ability to stimulate alloreactive T cells, including reduced production of IFNγ - Inhibition of the differentiation of monocytes to DCs by the interaction of C1q with LAIR-1 - Inhibition of INFα secretion by pDCs by the interaction of C1q with LAIR-1	(85-87, 140)
Lymphocytes	- Binding of sC1q to B and T cells in a dose-dependent manner - Inhibition of mitogen-induced T cell proliferation - Stimulation of Ig production in B cells (S. aureus activated) derived from healthy donors and 2 SLE patients in a dose-dependent manner	(138, 141, 142)

Neutrophils	- Upregulation of CR3 expression and adherence to albumin-coated surfaces by stimulated neutrophils - Stimulation of migration (mainly chemotaxis and not chemokinesis) in a dose-dependent manner → mediated by the CLR of C1q	(143, 144)
Fibroblasts	- Activation of C1qRs leads to Ca^{2+}-influx and stimulation of Ca^{2+}-activated K^{+} channels and initiation of chemotaxis of human skin fibroblasts - Response of proliferating fibroblast to sC1q: → Increased phosphorylation of p38 MAPK in proliferating fibroblasts partially mediated by the binding of CLR to CRT → Induction of apoptosis by CLR in fibroblasts	(145, 146)

Table 3.2: Papers analyzing the effect of imC1q.

Cell type	C1q-mediated effect	Ref
Monocytes	- Upregulation of phagocytosis of SRBCs by imC1q-primed monocytes - Modulation of LPS-induced cytokine production at the mRNA and protein levels towards a less inflammatory response: → suppression of proinflammatory cytokines: IL-1α/β, TNFα → upregulation of IL-10, IL-1RA, MCP-1, IL-6	(24, 135, 147)
HMDMs	- Modulation of LPS-induced cytokine release towards a less inflammatory response - Upregulation of FcγR- and CR-mediated phagocytosis of apoptotic cells - Upregulation of C3 secretion - Enhanced phagocytosis of SRBCs by imC1q-primed HMDMs	(24, 138, 147)
DCs	- Modulation of LPS-induced cytokine release towards a less inflammatory response - Induction of maturation of iDCs: → Upregulation of MHC class II, CD80, CD83, CD86, and CCR7 expression → Elevated secretion of IL-12, IL-10, and TNFα → Increased T cell stimulating capacity (increased production of IFN-γ consistent with the generation of a Th1 response) → Induces NF-κB translocation to the nucleus	(24, 86)
Neutrophils	- C1q-stimulated superoxide production: → Stimulation of O^{2-} release via a Ca^{2+}-dependent pathway → Costimulatory signal by CD18 (beta2 integrins) binding to ICAM-1 essential for C1q-triggered superoxide production → Inhibition of C1q-triggered superoxide production by blocking CD59	(148-150)
T cells	- Regulation of IFN-γ, IL-4, and IL-10 production by CD3-positive T cells	(83)

Table 3.3: Papers analyzing the effect of anti-C1q bound to imC1q.

Cell type	C1q-mediated effect	Ref
Monocytes	- Induction of proinflammatory cytokine response (IL-1β, IL-6, TNFα: ↑; IL-10: ↓)	(unpub. data)
HMDMs	- Induction of a proinflammatory phenotype in macrophages: → Induction of proinflammatory cytokine response (IL-1β, IL-6, TNFα: ↑; IL-10: ↓) → Upregulation of MHC class II, CD14, CD80, and CD274 expression → Downregulation of CD163 and MerTK → Downregulation of FITC-dextran uptake as compared to imC1q → Downregulation of phagocytosis rate of apoptotic cells - Elevated C1q secretion	(S. Thanei et al. (sub.))
DCs	- Elevated C1q secretion (iDCs>>mDCs)	(unpub. data)

Table 3.4: Papers analyzing the effect of C1q bound to the surface of apoptotic cells (imC1q on apoptotic cells).

Cell type	C1q-mediated effect	Ref
Monocytes	- Modulation of LPS-induced cytokine release towards a less inflammatory response during the ingestion of apoptotic cells	(24)
HMDMs	- Opsonization of apoptotic cells by C1q and facilitation of apoptotic cells uptake - Modulation of LPS-induced cytokine release towards a less inflammatory response during ingestion of apoptotic cells - Stimulation of the phagocytosis of apoptotic cells by ligation of CRT (cC1qR)/CD91 → involvement of macropinocytosis - Opsonization of apoptotic cells by C1q induces ERK signaling - C1q binding to CRP-opsonized apoptotic cells: → Activation of early (only CP) but not late complement components and promoting phagocytosis → Ingestion of apoptotic cells by macrophages stimulated TGF-β secretion (non-inflammatory clearance of apoptotic cells) - Directing of macrophage polarization during the uptake of apoptotic cells: → Modulation of gene expression in LPS-stimulated macrophages associated with: JAK/STAT signaling, chemotaxis, immuno-regulation, and NLRP3 inflammasome activation → Upregulation of LPS-induced cytokine release (type I IFN, IL-27, IL-10) → Inhibition inflammasome activation: down-regulation of procaspase-1 cleavage and caspase-1-dependent cleavage of IL-1β - Regulation of T cell proliferation: → Upregulation of PD-L1/2 and CD40 expression → Reduction of allogenic and autologous Th17 and Th1 subset proliferation → Increased Treg proliferation	(22-25, 137, 151, 152)

DCs	- Increased phagocytosis rate of apoptotic cells by iDCs → Increased secretion of IL-12 in the presence of LPS (Inhibition by PTX3) - Opsonization of apoptotic cells by C1q and facilitation/enhancing of apoptotic cells uptake → in a C1q dose-dependent manner → stimulation of IL-6, IL-10, TNFα but no effect on IL-12p70 secretion - Modulation of LPS-induced cytokine release towards a less inflammatory environment during ingestion of apoptotic cells - Regulation of T cell proliferation: → Upregulation of PD-L2 and CD86 expression → Reduction of autologous Th17 and Th1 subset proliferation	(23, 24, 122, 137)

Table 3.5: Papers analyzing the effect of anti-C1q bound to imC1q on apoptotic cells.

Cell type	C1q-mediated effect	Ref
THP-1 cells	- Anti-C1q target C1q deposited on the surface of early apoptotic cells → suppression of the uptake of apoptotic cells by THP-1 cells	(153, 154)

Table 3.6: Papers analyzing the effect of C1q bound to other surfaces.

Cell type	C1q-mediated effect	Ref
Monocytes	- Interaction of C1q with LDL: → C1q binding to modified LDL but not to native LDL → Enhanced ingestion of LDL upon C1q binding to LDL → Upregulation of CD80 and CD31 expressions → Upregulation of CCL2 chemokine expression	(155)
HMDMs	- C1q binding to modified LDL but not to native LDL: → Upon C1q binding to LDL enhanced ingestion - Engulfment of NETs by HMDMs in a cytochalasin D-dependent manner → active, endocytic process - C1q binding and opsonization of NETs facilitates their uptake by HMDMs → silent process	(155, 156)

Table 3.7: Papers analyzing the effect of C1q-bearing ICs (C1q-ICs).

Cell type	C1q-mediated effect	Ref
Monocytes	- Incubation of monocytes with C1q-ICs derived from SLE patients: → Reduction of the expression of IFN-response genes → No detectable upregulation of CD40 and CD86 expression - C1q promotes the binding of SLE ICs to monocytes → Altering the trafficking of ICs within monocytes: ICs persisted in early endosomes → Inhibition of IFNα secretion by C1q-ICs	(157, 158)
DCs	- C1q slightly promotes the binding of SLE ICs to pDCs	(157)

T cells	- Effect of C1q-ICs: → Stimulation of TNFα and IFNγ secretion in a dose-dependent manner → Upregulation of CD25 (low expression levels)	(159)

Legend: CRP: C-reactive protein; CRT: Calreticulin; ERK: extracellular signal-regulated kinase; IL-1RA: IL-1 receptor antagonist; JAK: Janus kinases; LAIR-1: leucocyte-associated Ig-like receptor 1; LDL: lipoproteins; M1: proinflammatory macrophages; M2: anti-inflammatory macrophages; MAPK: mitogen-activated protein kinase; MCP-1: monocyte chemoattractant protein-1; MerTK: Mer tyrosine kinase; NF-κB: nuclear factor- κB; pDCs: plasmacytoid DCs; PD-L1/2: programmed cell death ligand-1/2; PTX3: pentraxin 3; STAT: signal transducers and activators of transcription

Anti-C1q

Hypocomplementemia, that most SLE patients display during a disease flare, is believed to be rather due to an accelerated consumption rate of complement proteins by aberrant complement activation and not the consequence of a primary deficiency of complement components (6). A rational reason for low C1q levels are anti-C1q. These autoantibodies were first detected in 1971 (160) and finally identified in 1988 by Antes et al. and Uwatoko et al. (161, 162). Anti-C1q have been observed in patients suffering from different autoimmune diseases but also in patients with renal or infectious diseases as well as in healthy individuals (Table 4). The incidence of anti-C1q varies highly between these different diseases. In a healthy population, anti-C1q positive individuals varied between 4-10% depending on the age of the population analyzed (163-165). In unselected SLE patients, anti-C1q positivity has been found to range from 20-50% (7-9). Nevertheless, the highest anti-C1q levels were found in patients suffering from hypocomplementemic urticarial vasculitis syndrome (HUVS) with a penetrance of 100% (166). No differences in the binding characteristics of anti-C1q between SLE or HUVS patients could be identified (167).

Table 4: List of selected pathological conditions associated with the occurrence of anti-C1q.

	Disease	**Anti-C1q occurrence** [%]	**Ref**
autoimmune diseases	HUVS	100%	(166)
	SLE	20-50%	(7-9, 29, 163, 166, 168)
	- without lupus nephritis	- 14-32%	
	- with lupus nephritis	- 60-100%	
	Sjörgen's syndrome	13%	(169)
renal diseases	membranoproliferative glomerulonephritis	54-88%	(14, 170)
	IgA nephropathy	0-31%	(14, 171)
infectious diseases	HIV	13%	(172)
	hepatitis C	26%	(164)
-	healthy individuals	4-10%	(163-165)

The strong association of anti-C1q with lupus nephritis suggests that these autoantibodies have a major role in the pathogenesis of lupus nephritis (7-9). Even though, anti-C1q are associated with renal involvement, direct evidence how these autoantibodies contribute to renal damage is not yet available. A study in a lupus-prone mouse model suggests that renal inflammation is only induced by anti-C1q in combination with preformed glomerular C1q-containing ICs (10). The authors suggested that anti-C1q triggered complement activation is determined by the amount of deposited ICs and C1q in the kidneys. They hypothesized that, if complement activation passes beyond a certain threshold level, complement activation and

the infiltrating inflammatory cells lead to cell damage in the kidneys with consecutive overt renal disease and loss of renal function (10).

Figure 5 illustrates the proposed mechanism of disease: ICs deposition occurs along the glomerular basal membrane in the kidneys of patients suffering from a pre-existing ICs-glomerular nephritis (Figure 5A). C1q from the circulation or locally produced by infiltrating myeloid cells can then be fixed by these ICs (C1q-ICs) (Figure 5B). Due to the tight regulation of the complement activation by complement regulators, it is believed that this construct has not yet the ability to efficiently activate the complement cascade. Nevertheless, C1q-ICs can then be targeted by anti-C1q (Figure 5C). If sufficient C1q-ICs are deposited in the kidneys, bound anti-C1q can trigger further complement activation which is not sufficiently inhibited by complement regulators anymore. This will induce full-blown complement activation and secondary infiltration and activation of inflammatory immune cells, which ultimately lead to kidney damage (Figure 5D). With regard to downstream mechanisms, anti-C1q are believed to involve both, activation of the complement system as well as recruitment of FcγRs upon binding to, e.g., C1q-ICs deposited in the kidneys, with consecutive amplification of the inflammatory process during flares (10).

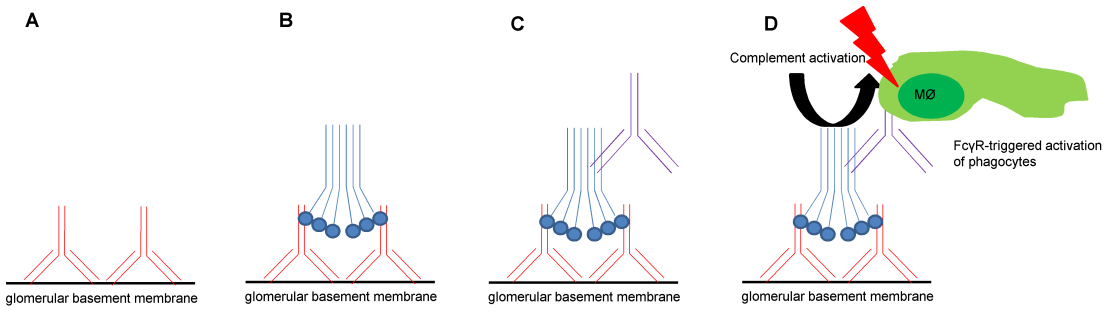


Figure 5: The pathogenic effect of anti-C1q in nephritic kidneys of SLE patients.

ICs deposit along the glomerular basement membrane in the kidneys of SLE patients (A). C1q is fixed onto these deposited ICs (B). Upon binding of C1q to deposited ICs, C1q exposes a cryptic epitope to which anti-C1q can bind to (C). Activation of the complement system and infiltration and activation of inflammatory immune cells leads to inflammation and tissue damage (D). The pathogenic mechanism of anti-C1q is thought to require both activation of the complement system as well as the recruitment of FcγRs for their pathogenicity (modified according to (10)).

Importantly, the mechanism which renders C1q antigenic and leads to the production of anti-C1q has not yet been identified. However, the occurrence of anti-C1q was demonstrated to be Ag-driven using phage display technology (173). Anti-C1q bind with high-affinity via their $F(ab')_2$ fragments to the CLR of C1q but not to the globular heads (161, 174). Furthermore, anti-C1q bind to a cryptic epitope exposed on surface-bound C1q, but hidden on fluid-phase

C1q (175, 176). Recently, a specific linear epitope (termed A08), located on the C1qA chain, could be identified as a major target of anti-C1q (177). Nevertheless, this does not exclude the existence of additional epitopes. Anti-C1q are specifically directed against C1q since no cross-reactivity with structural similar collectins, such as MBL or lung surfactant protein A, could be detected (178, 179). *In vitro* experiments evaluating the binding characteristics of anti-C1q demonstrated that anti-C1q do not recognize C1q when it is bound to different IgG-classes or ICs, suggesting that none of these constructs are able to present C1q and, more importantly, the cryptic epitope that is required for the binding of anti-C1q (153). Therefore, alternatively to the mechanism proposed in Figure 5, it has been hypothesized that these autoantibodies might rise due to a decreased or aberrant clearance of dying cell material which has been shown to occur in SLE patients (reviewed in (50)). Indeed, anti-C1q target C1q bound to the surface of early apoptotic cells (153, 154). This might induce autoantibody formation due to the prolonged exposure of C1q epitopes which are normally hidden in fluid-phase C1q. In this context, it has been hypothesized that the binding of anti-C1q to C1q might interfere with the process of phagocytosis. Indeed, Pang et al. demonstrated that anti-C1q of lupus nephritis patients bind to C1q deposited on the surface of early apoptotic cells and thereby suppressed their removal by THP-1 cells (154).

Anti-C1q can be of the IgG-, IgA-, or IgM-class (14). However, the IgG-class distribution is discussed controversially. Whereas Coremans et al. and others mainly found IgG2 and IgG3 (15, 167), other groups found IgG1 and IgG2 (180-182). Depending on the anti-C1q subclass, these autoantibodies might differ in their pathogenic properties, based on their interaction with bound C1q, concerning complement activation and FcγR activation on phagocytes. Indeed, we found that SLE patient-derived anti-C1q amplify complement activation *in vitro* via the CP and the LP dependent on the anti-C1q subclass repertoire present in the lupus patient's serum (183) (see part 1, p. 29). In addition, we can show that imC1q-bound anti-C1q alter the C1q-dependent suppression of macrophage-mediated inflammation by inducing a proinflammatory phenotype via an FcγRII-dependent pathway and induce C1q secretion by HMDMs (S. Thanei et al. (both manuscripts submitted); see parts 2/3, p. 46/73).

Macrophages

The classification of macrophage subsets

Macrophages are key players of the innate immune system. They participate in host defense, clearance of dying and dead cells, tissue remodeling and homeostasis, inflammatory processes, and in immune diseases by contributing to tissue damage and pathology (184).

The functional profile of macrophages is determined by their activation and exposure to environmental factors, such as cytokines and growth factors, during their differentiation from monocytes into macrophages (185). In analogy to the Th cell nomenclature, Mantovani et al. proposed a concept for dividing macrophages in a continuum between 2 functionally polarized states based on the cytokines used for in vitro polarization (Table 5) (184). *In vitro*, monocytes can be polarized into different macrophage subsets by specific cytokines, the most prominent being GM-CSF, IFN-γ, or LPS to induce mainly proinflammatory macrophages (termed M1), or M-CSF or IL-10 to induce mainly anti-inflammatory macrophages, also called alternatively activated or anti-inflammatory macrophages (M2). M2 can be further divided in M2a, M2b, and M2c subsets, which are induced by IL-4 or IL-13, ICs in combination with toll-like receptor (TLR) ligands such as LPS, and IL-10, TGFβ, or Glucocorticoids (particularly Dexa), respectively. Interestingly, the stimuli used for M2 polarization are very heterogeneous. However, polarized macrophages exhibit functional differences evident by their phenotypic profiles such as cytokine release and surface markers (185-187). M1 macrophages mainly produce proinflammatory cytokines, phagocyte microorganisms and are often linked to tissue injury and inflammation, whereas M2 macrophages display in general a low Ag-presenting capacity, inhibit and prevent T cell activation, and are associated with tissue repair and fibrosis (188, 189).

Table 5: Overview of different macrophage subsets.

		M1 (classically activated macrophages)	**M2** (alternatively activated macrophages)
Polarizing cytokines used in *in vitro* experiments		GM-CSF IFNγ, LPS, TNFα,	M-CSF M2a: IL-4, IL-13 M2b: ICs + IL-1β/LPS M2c: IL-10, TGFβ, Glucocorticoids
Cytokine secretion profile		Proinflammatory cytokines (IL-1β, IL-6, IL-12, IL-15, IL-18, TNFα, …)	Anti-inflammatory cytokines (IL-10) Proinflammatory cytokines
Induction of T cell response		Th1 response	Th2 effector response/functions
Expression of surface markers	CD14	+++	+ (↓)
	CD40	++	+++
	CD80	+++	++
	CD86	+++ (↑)	+ (M2b: (↑); M2a: (↑))
	CD163	+	++++ (↑)
	CD206	+ (↓)	++ (↑)
	CD273	+	++
	CD274	+++	+
	MHC class II	+++ (↑)	+ (M2b: (↑))
Functions		Endocytosis Type I inflammation Killing of intracellular pathogens Tumor resistance	Type II inflammation Allergy Killing and encapsulation of parasites Tissue repair and remodeling Clearance of apoptotic cell material Promoting angiogenesis Wound healing
Metabolism characteristics		High levels of nitric oxide synthase	High levels of arginase
C1q secretion		High C1q secretion levels	Low C1q secretion levels

Summarized from: (190, 191)

The model used to categorize macrophages originally only referred to M1 and M2 subsets which are associated with different functions as mentioned above (188, 189). However, translation of the macrophage phenotypes, as described before, into disease models has been recognized to be over simplified. The classification of macrophages into the different subsets should be regarded as a useful framework. *In vivo*, macrophages are constantly encountering various signals. Therefore, it might be possible that macrophages exhibit a phenotype showing both M1 and M2 characteristics and that multiple phenotypes coexist in tissues. Additionally, polarization of macrophages is thought to be not permanent but rather milieu-dependent and partially reversible, i.e., in response to their microenvironment macrophages can express constantly changing phenotypes, also termed plasticity (184, 188, 192). In disease conditions, macrophages might play a dual role. Therefore, it is not surprising

that so far no characteristic macrophage phenotype could be defined in lupus nephritis. Different reports show that infiltrating macrophages/DCs in murine lupus nephritis are very heterogeneous (193, 194). Nevertheless, mononuclear cells play a role in the pathogenesis of organ related diseases, such as lupus nephritis, and are associated with chronic tissue damage and injury (195).

Macrophages and DCs in SLE patients

Functional defects and abnormalities in the cells of the monocyte-macrophage lineage obtained from SLE patients are well known, although the underlying molecular mechanism is not fully understood. Macrophages obtained from SLE patients exhibit a defect in phagocytosis of apoptotic cell material correlating with low complement levels (17, 18). Additionally, monocytes from SLE patients have an abnormal cytokine secretion profile in response to apoptotic cells independent of the monocyte's phagocytic efficiency or the patient's disease state (19). This contributes to the accumulation of a large number of apoptotic cells which can be found in various tissues (20, 21). Further functional alterations occurring in SLE patient-derived cells are listed in Table 6.

Table 6: Functional alterations in monocytes, macrophages, and DCs from SLE patients.

		Monocytes	Macrophages	DCs
Phagocytosis capability		↓ Phagocytosis rate ↑ TNFα/↓TGFβ during uptake (19)	↓ Phagocytosis rate (17, 196)	na
Cytokine secretion		↑ TNFα (197) ↑ IL-10	↑ IL-10 (S. Thanei et al. (submitted))	↑ IL-8 (198)
Chemotaxis		↑ (199)	na	na
Expression of surface markers		↑ CD64 on circulating monocytes (199) ↑ CD40 (200) ↓ CD14 (197)	na	Inability to mature (201) ↑ CD80, CD86, HLA-DR (198)
T cell proliferation induction		na	na	↑ Proliferation and activation of allogenic T cells (198)
C1q secretion	C1q mRNA	Unstimulated: normal Stimulated: ↓C1q expression (99)	nt	nt
	C1q secretion	nt	Normal (S. Thanei et al. (submitted)) ↑ LMW-C1q (132-134)	nt
Apoptosis		na	↑ (196)	na
Superoxide production		↓ (202)	na	na

Legend: na: not available; nt: not tested

Based on the characteristic features of M1/M2 macrophages one would expect that SLE patients display a predominance of M1-like cells. Low phagocytosis ability of apoptotic cells and high secretion rates of proinflammatory cytokines (such as TNFα) would underline this assumption. Nevertheless, the M1 versus M2 paradigm may not be appropriate for the classification of lupus cells. M1 macrophages are unlikely to produce IL-10 which can be found at high levels in lupus patients. These high IL-10 levels are considered to be a hallmark of the disease and were found to correlate with disease activity (203, 204). Furthermore, the combination of ICs and TLR ligands favor the induction of M2b macrophages. Studies of lupus prone mice suggest that both macrophage subtypes, M1 as well as M2, are increased in lupus nephritis kidneys (193, 194, 205).

Part 1

Anti-C1q autoantibodies from systemic lupus erythematosus patients activate the complement system via both the classical and lectin pathways

Abstract

Autoantibodies against complement C1q (anti-C1q) strongly correlate with the occurrence of lupus nephritis and hypocomplementemia in systemic lupus erythematosus (SLE). Although a direct pathogenic role of anti-C1q has been suggested, the assumed complement-activating capacity remains to be elucidated. Using an ELISA-based assay, we found that anti-C1q activate the classical (CP) and lectin pathways (LP) depending on the anti-C1q immunoglobulin-class repertoire present in the patient's serum. IgG anti-C1q resulted in the activation of the CP as reflected by C4b deposition in the presence of purified C1 and C4 in a dose-dependent manner. The extent of C4b deposition correlated with anti-C1q levels in SLE patients but not in healthy controls. Our data indicate that SLE patient-derived anti-C1q can activate the CP and the LP but not the alternative pathway of complement. These findings are of importance for the understanding of the role of anti-C1q in SLE suggesting a direct link to hypocomplementemia.

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by B cell hyperactivity, antibodies specific for various auto-antigens, the formation of immune complexes (ICs), the aberrant activation of the complement system resulting in complement deposition and hypocomplementemia (44, 45).

The complement system is an integral part of the innate immune system, consisting of several plasma and cell-associated proteins, and acting upon triggering as a cascade resulting in the opsonization and lysis of targeted cells (e.g., pathogens), in the production of anaphylatoxins (C3a, C5a), and in the recruitment of immune cells to the site of local inflammation (1). The complement system can be activated by at least 3 different pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP) (1, 2). Each of these pathways is initiated by a specific ligand. The CP is activated by binding of the C1 complex to ICs, the LP by binding of mannose-binding lectin (MBL) or ficolins to, e.g., mannose-containing groups on bacterial surfaces, and the AP by spontaneous C3(H_2O) binding and activation/turn-over or by bacterial products (e.g., lipopolysaccharide (LPS)) (1, 2). All 3 pathways converge in the formation of the C3 convertase, the cleavage of C3 into C3a and C3b, further downstream activation of terminal complement components and finally in the assembly of the membrane-attack complex (C5b-C9) (35).

Primary deficiencies of early components of the CP (C1q, C4, C2) are strongly associated with SLE. In particular, homozygous C1q deficiency was shown to be the strongest genetic susceptibility to develop SLE (3, 4). This observation suggests that complement plays a major role in the pathogenesis of SLE. However, most SLE patients do not suffer from primary C1q deficiency, but aberrant complement activation is accounted for low complement levels (5). A rational reason for low C1q levels are autoantibodies against C1q (anti-C1q) that are present in 20-50% of unselected SLE patients. Anti-C1q levels in these patients not only correlate with hypocomplementemia but also with the occurrence of proliferative lupus nephritis (7-9). Limited evidence is available supporting a direct role of these autoantibodies in the pathogenesis of lupus nephritis. Animal models suggest that renal inflammation is not induced by anti-C1q deposited in the kidneys together with C1q alone. Anti-C1q were only found to be pathogenic in these models in combination with preformed glomerular C1q-containing ICs (10). Anti-C1q were shown to bind to a cryptic epitope only exposed when C1q is surface-bound, but that is shielded on C1q in fluid-phase (161). Importantly, anti-C1q specifically target C1q bound to early apoptotic cells (153, 154), providing a link between SLE, C1q, and apoptosis.

Little is known about the complement-activating potential of anti-C1q bound to immobilized C1q (imC1q) or which activation pathways might be triggered by anti-C1q. In a recent study, Pang et al. demonstrated that affinity purified anti-C1q of lupus nephritis patients inhibited the activation of the CP using ICs as the initiating structure (154). In contrast, other studies showed that autoantibodies in general have rather complement-activating capacities (206). However, the complement pathway that is primarily triggered by autoantibodies seems not only to be dependent on the dominant immunoglobulin-class (Ig-class) but also on the target antigen. In an *in vitro* assay, anti-citrullinated protein antibodies from rheumatoid arthritis (RA) patients were found to activate both, the CP as well as the AP of complement (207), whereas anti-neutrophil cytoplasmic autoantibodies were described to primarily activate the AP without apparent signs of LP or CP activation (208). Others demonstrated that ICs consisting of collagen and anti-collagen autoantibodies could activate both the CP and the AP (209). Also, anti-phospholipid antibodies have been linked to complement activation whereby the CP was found to be the initiator and the AP the amplifier of complement activation (210). Finally, cryoglobulins were shown to activate all 3 pathways of complement, the CP, the LP, and the AP (211).

The aim of this study was to elucidate the complement-activating potential of SLE patient-derived high-affinity anti-C1q. Comprehension of the complement-activating potential of anti-C1q will improve the understanding of the disease and support the development of complement-targeting treatments.

Patients and methods

Anti-C1q source

A cohort of 27 SLE patients (Table 7) and 25 healthy control donors were included in the study.

Table 7: SLE patient characteristics.

Sex (females/males)	22/5
Age (mean range years)	47 (28-71)
Hypocomplementemia (low C3 and C4) (yes/no) [1]	14/13
Lupus nephritis (yes/no) [1]	16/11
Positive for anti-C1q[1]:	20
- Positive for IgG anti-C1q	9
- Positive for IgG and IgM anti-C1q	3
- Positive for IgG and IgA anti-C1q	5
- Positive for IgG, IgM, and IgA anti-C1q	3
Negative for anti-C1q[1]	7

[1] Information at time point of blood sampling

All SLE patients fulfilled at least 4/11 criteria of the American College of Rheumatology (212, 213). Collection and use of serum samples were approved by the local Ethics Committee (EKZ-No.: 110/04; 130/05). Before serum samples were diluted to their final working concentration, each sample was heat-inactivated (56°C, 30min) and centrifuged at 14'000xg for 30min at 4°C.

Complement-active/-deficient sera and dilution buffers

As a source of complement, normal human sera (NHS) from healthy donors were aliquoted and stored at -80°C until used. These NHS had normal C1q and MBL levels and no detectable anti-C1q levels. In addition, we used MBL-deficient sera from healthy donors (undetectable MBL; but normal C1q levels) and C1q-depleted serum (C1qDS; Complement Technology: negative for C1q; but normal MBL levels).

Purified complement proteins and sera used as a source of complement were diluted either using veronal buffered saline (VBS: 5mM barbituric acid/0.5mM $MgCl_2$/2mM $CaCl_2$/140mM NaCl/0.05% Tween, pH 7.5), Mg-EGTA buffer (modified VBS: 10mM EGTA/15mM $MgCl_2$/140mM NaCl/0.05% Tween, pH 7.5), or PBS-EDTA (PBS/10mM EDTA/0.05% Tween, pH 7.5). The presence of 0.05% Tween did not impair the activation of any of the complement pathways (data not shown).

Detection of anti-C1q levels

For the detection of anti-C1q levels, ELISA plates (MaxiSorp, Nalge Nunc International) were coated with purified C1q (5µg/ml; Complement Technology) in coating buffer (0.1M sodium carbonate buffer, pH 9.6) overnight at 4°C. After each incubation step, the plates were washed 3times with washing buffer (PBS-T: PBS/0.05% Tween 20). C1q-coated plates were incubated with serum samples from SLE patients or healthy donors diluted 1:50 in high-salt buffer (HS-PBS: PBS-T/1M NaCl) for 1h at 37°C. After washing, different anti-C1q classes (IgG, IgA, IgM) were detected with specific antibodies diluted in HS-PBS for 1h at room temperature (RT). Bound IgG was detected with alkaline phosphatase (AP)-conjugated rabbit anti-IgG (Promega), bound IgA with AP-conjugated goat anti-IgA, and bound IgM with AP-conjugated donkey anti-IgM (both obtained from Jackson ImmunoResearch). The enzyme activity of AP was detected by incubating plates with AP substrate (Sigma-Aldrich) according to the manufacturer's instructions. Absorbance was read at 405nm using a microplate biokinetics reader (BioTek instruments). For data analysis the results were standardized as follows: measurements were expressed in units relative to the O.D. values of a reference SLE serum having high anti-C1q levels and corresponding to 1'000 arbitrary units (AU). The reference serum was used as an internal control and included on each plate in each experiment. The cut-off was determined as the interquartile mean (IQM) of the AU obtained with NHS plus 3times the SD. Every sample was tested in duplicate within a single experiment, and experiments were performed 3times.

Complement activation assays

Detection of CP, LP, and AP activation

Functional activity of the 3 complement activation pathways was analyzed by ELISA using coated IgM (2µg/ml) for the CP, coated mannan (100µg/ml; *Saccharomyces cerevisiae*; M7504) for the LP, and coated LPS (10µg/ml; *Escherichia coli*: 0127:B8, all obtained from Sigma-Aldrich) for the AP. Plates were coated over night at 4°C. Residual binding sites were blocked with blocking buffer (BSA-PBS: PBS/1% BSA) for 1h at RT. Then, the plates were washed and incubated for 1h at 37°C with NHS diluted at 1% (for the CP and the LP) or 10% (for the AP) in VBS, Mg-EGTA buffer, or PBS-EDTA buffer as a source of complement. After a wash step, deposited C3 was detected by goat anti-C3 (Quidel) and deposited C4b by goat anti-C4b (Complement Technology). For both a secondary HRP-labeled mouse anti-goat IgG (Sigma-Aldrich) was used. After a final washing step, HRP activity was quantified by using TMB substrate (BD Biosciences) according to the manufacturer's instructions. The reaction was stopped by the addition of 4M H_2SO_4 and absorbance was read at 450nm.

Complement activation by anti-C1q

To investigate the ability of anti-C1q in activating the complement system, ELISA plates were coated with C1q in coating buffer overnight at 4°C and free binding sites were blocked with PBS-BSA for 1h at RT. After a washing step, plates were incubated for 10min at 37°C with heat-inactivated sera from SLE patients or healthy donors diluted in HS-PBS. This short 10min incubation time was found to be sufficient for anti-C1q binding to imC1q in preliminary kinetic experiments (data not shown). Then, plates were washed again. In case of using sera as a source of complement for the assay, plates were incubated for 1h at 37°C with complement-active NHS or complement-deficient sera (MBL-deficient serum/C1qDS) diluted 1 or 10% in VBS or Mg-EGTA buffer respectively. For the CP-specific assay, plates were incubated for 1h with purified C1 (0.5µg/ml) diluted in VBS, followed by an incubation of 40min with purified C4 (2µg/ml; both obtained from Complement Technology) diluted in VBS. After a wash step, activation of complement was assessed by detecting deposited C4b or C3 fragments using specific antibodies as described before.

Statistical analysis

Data are expressed as AU or as mean (O.D. values) ± SEM. The significance of differences between 2 groups was determined using the Mann-Whitney U test and correlations were calculated using the Spearman's rank correlation coefficient. More than 2 groups were compared using one-way ANOVA followed by the Bonferroni post-test (GraphPad Prism). A $p<0.05$ was considered statistically significant.

Results

Detection of *in vitro* complement activation

To analyze the complement-activating capacity of patient-derived anti-C1q *in vitro*, an ELISA-based assay was developed to specifically distinguish the activation of the CP, the LP, and the AP. Selective inhibition of specific complement pathways was obtained by using special buffers, lacking either magnesium and/or calcium, or by using complement-deficient sera.

NHS diluted in VBS, a buffer containing both calcium and magnesium, allowed the activation of all 3 complement pathways evident by both C4b and C3 depositions. When NHS was diluted in Mg-EGTA buffer, containing only magnesium but no calcium, clear C3 deposition could be measured indicating AP activation, whereas only minor C4b deposition could be observed on LPS coating. Finally, using EDTA buffer, devoid of both calcium and magnesium, prevented the activation of all three pathways (Figure 6A+B).

The specificity of this assay was confirmed by using C1qDS or MBL-deficient serum instead of NHS as a source of complement (Figure 6C+D). As expected, MBL-deficient serum showed normal CP activity but no LP activity. C1qDS showed no CP activity. However, C1qDS had in general lower complement activity than NHS potentially as a consequence of the C1q-depleting manipulation.

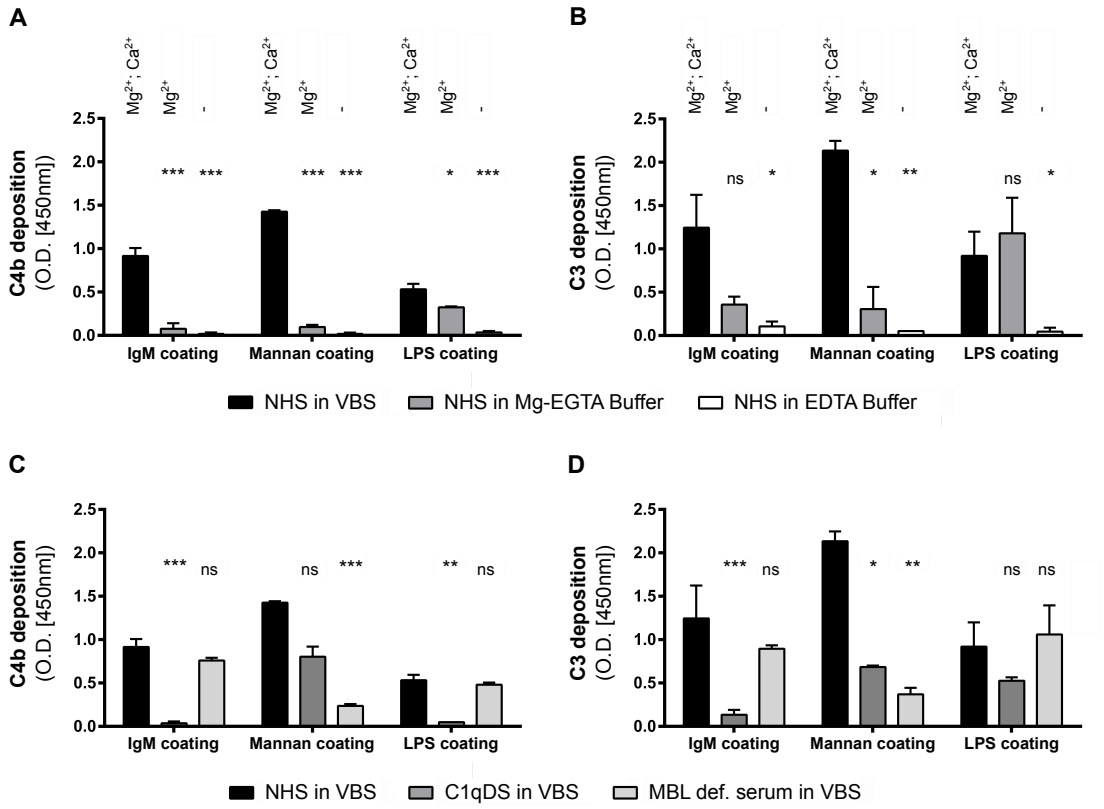


Figure 6: Activation of the 3 pathways of complement in vitro.

IgM-, mannan-, or LPS-coated plates were used for the activation of the CP, the LP, or the AP. Coated plates were incubated with NHS, as a source of complement, diluted in special buffers (VBS, Mg-EGTA, or EDTA-PBS) and either C4b deposition (A) or C3 deposition (B) was detected. Under the same coating conditions, plates were also incubated with C1qDS or MBL-deficient serum and again C4b deposition (C) as well as C3 deposition (D) were measured. Data represent the mean ± SEM of 3 independent experiments. One-way ANOVA incl. Bonferroni post-test (vs VBS control), $p<0.05$*, $p<0.01$**, $p<0.001$***.
Legend: Mg^{2+}: magnesium; Ca^{2+}: calcium.

Anti-C1q activate the CP and the LP but not the AP

In order to study the complement-activating capacity of anti-C1q, we used C1q immobilized (imC1q) to a polystyrene surface and serum from either SLE patients or healthy donors as a source of anti-C1q. All sera were diluted in high-salt buffer. By the use of a high-ionic strength buffer containing 1M NaCl, three advantageous effects were achieved: avoiding unspecific ICs binding to imC1q, allowing only binding of high-affinity anti-C1q to imC1q, and blocking activation of complement factors present in SLE serum (data not shown; (14, 174)). Complement activation was further inhibited by heat-inactivation of all sera which did not affect the binding of anti-C1q to imC1q (data not shown).

To validate the assay, four different SLE patients and two healthy donors were selected based on the Ig-class of anti-C1q present in their sera. SLE(1) had IgG and IgA anti-C1q, SLE(2) IgG and IgM anti-C1q, and SLE(3) IgG, IgA, and IgM anti-C1q, whereas SLE(4) and both healthy donors (NHS(1+2)) were negative for anti-C1q. After incubation of the sera on C1q-coated plates, NHS was added as a source of complement and C4b and C3 depositions were quantified as described in patients and methods.

Although, there was a low amount of C3 and C4b deposition on imC1q after exposure to NHS that was independent of anti-C1q (SLE(4)/NHS(1+2)), we observed a significant enhanced deposition following the binding of anti-C1q to imC1q (Figure 7A+D). In order to discriminate whether C4 deposition was caused by the CP, the LP, or by both pathways, we next used MBL-deficient serum or C1qDS instead of NHS.

To evaluate the relative contribution of the CP, we used MBL-deficient serum diluted in VBS (Figure 7B). Yet, we could still detect C4b deposition, indicating that anti-C1q of the IgG- and IgM-class are able to activate the complement system via the CP. Anti-C1q-dependent CP activation was always higher in SLE patients having IgG and IgM anti-C1q (SLE(1–3)) as compared to anti-C1q negative individuals (SLE(4)/NHS(1+2)).

When instead CP activation was prevented by using C1qDS diluted in VBS, we could still see enhanced C4b deposition, suggesting that in the presence of NHS both, the CP and the LP, contribute to anti-C1q-mediated C4b deposition (Figure 7C). C4b deposition was evident in the presence of anti-C1q derived from patients having IgA anti-C1q (SLE(1+3)). But, low levels of deposited C4b could be also detected with SLE(2), a patient having no IgA anti-C1q. Control sera, SLE(4) and both healthy donors (NHS(1+2)), who were screened negative for anti-C1q, led to only minor C4b deposition.

Activation of all 3 complement pathways leads to the generation of deposited C3 fragments. Since anti-C1q from patients (SLE(1-3)) bound to imC1q induced levels of C3 deposition that were higher as compared to serum from anti-C1q negative patients or healthy donors

(SLE(4)/NHS(1+2) (Figure 7D)), we determined the relative contribution of the AP to this anti-C1q induced complement activation.

As seen in Figure 7E, C3 deposition was completely abolished when NHS was diluted in Mg-EGTA instead of VBS, suggesting that anti-C1q derived from SLE patients do not directly activate the AP.

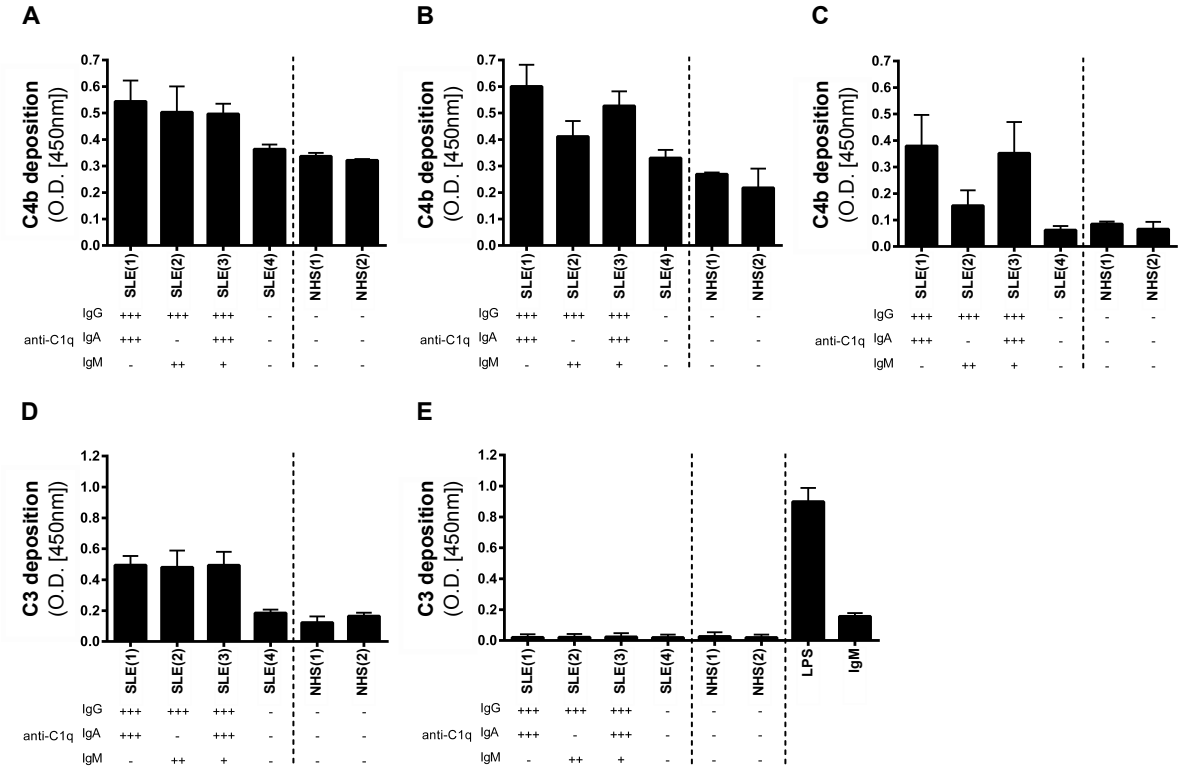


Figure 7: Anti-C1q bound to imC1q trigger the activation of the CP and the LP but not of the AP.

C1q-coated plates were incubated with sera of different SLE patients (3 anti-C1q positive, 1 anti-C1q negative) or sera of 2 different healthy donors (both anti-C1q negative). Subsequently, NHS diluted in VBS (A+D), MBL-deficient serum diluted in VBS (B), C1qDS diluted in VBS (C), or NHS diluted in Mg-EGTA buffer (E) was added and either C4b (A-C) or C3 (D+E) deposition was detected. Anti-C1q classes present in the serum sample are indicated for each patient and healthy donor below the figure (-: 0-250AU; +: 250-500AU; ++: 500-750AU; +++: 750-1'000AU). Data are shown as the mean ± SEM of 3 independent experiments.

Taken together, our data indicate that anti-C1q bound to imC1q activate the complement system via the CP and the LP depending on the anti-C1q class present in the patient's serum leading to C4b and C3 depositions. However, anti-C1q do not directly activate the AP.

Anti-C1q activate the CP and the LP dependent on the anti-C1q class

To further dissect the relative contribution of the CP and the LP in the complement activation mediated by anti-C1q, we screened a larger cohort of SLE patients and healthy donors. Occasionally healthy donor serum contains anti-C1q (29). Thus, we included 2 of such sera in the assay.

We used either MBL-deficient serum or MBL-deficient serum reconstituted with 4µg/ml purified MBL in addition to C1qDS or NHS. No C4b deposition could be measured on a plate coated with mannan alone using MBL-deficient serum, whereas the reconstitution of MBL-deficient serum with purified MBL partially restored LP activity. Likewise, the incubation of an IgM-coated plate with C1qDS did not lead to C4b deposition (Figure 8A).

Anti-C1q bound to imC1q were further incubated with NHS, C1qDS, or MBL-deficient serum with or without exogenous MBL (Figure 8B-D).

As shown in Figure 8B, C4b deposition triggered by anti-C1q bound to imC1q in the presence of NHS was higher in SLE patients and healthy donors being anti-C1q positive, independent of the anti-C1q class, as compared to anti-C1q negative individuals. However, as already observed in experiments outlined before, there was already significant C4b deposition, when C1q-coated plates were incubated with sera from anti-C1q negative SLE patients (SLE(4+10)) or healthy donors (NHS(1+2)).

In a next step, bound anti-C1q from the same individuals were further incubated with C1qDS allowing only LP-mediated complement activation (Figure 8C). No or little C4b deposition was detectable in patients and healthy donors having no anti-C1q (SLE(2+10)/NHS(1+2)). Interestingly, an association could be seen between LP-mediated C4b deposition and the presence of IgA anti-C1q (SLE(1/3/7/9)). Decreased C4b deposition was most pronounced when in addition to the absence of IgA anti-C1q the sera only contained either IgG or IgM anti-C1q (SLE(6+5)). Highest LP-mediated C4b deposition was observed with anti-C1q from patients (SLE(1/3/7)) or healthy donors (NHS(3+4)) consisting of mainly IgA or IgA combined with IgG/IgM anti-C1q. Together, these data suggest that IgA anti-C1q and to a lesser extend IgM anti-C1q are responsible for LP-mediated complement activation.

In analogy, plates with anti-C1q bound to imC1q were incubated with MBL-deficient serum with or without exogenous MBL (Figure 8D). As expected, no restoration of C4b deposition was observed for sera being anti-C1q negative or having only IgG anti-C1q (SLE(4/5/10)/NHS(1/2)) when using MBL-deficient serum supplemented with exogenous MBL. In contrast, in SLE patients having IgA anti-C1q (SLE(1/3/7/9)), C4b deposition was higher, when we used MBL-deficient serum reconstituted with MBL as compared to MBL-deficient

serum alone. We also observed an increased MBL-dependent C4b deposition triggered by IgM anti-C1q in SLE patients (SLE(2/6/8)).

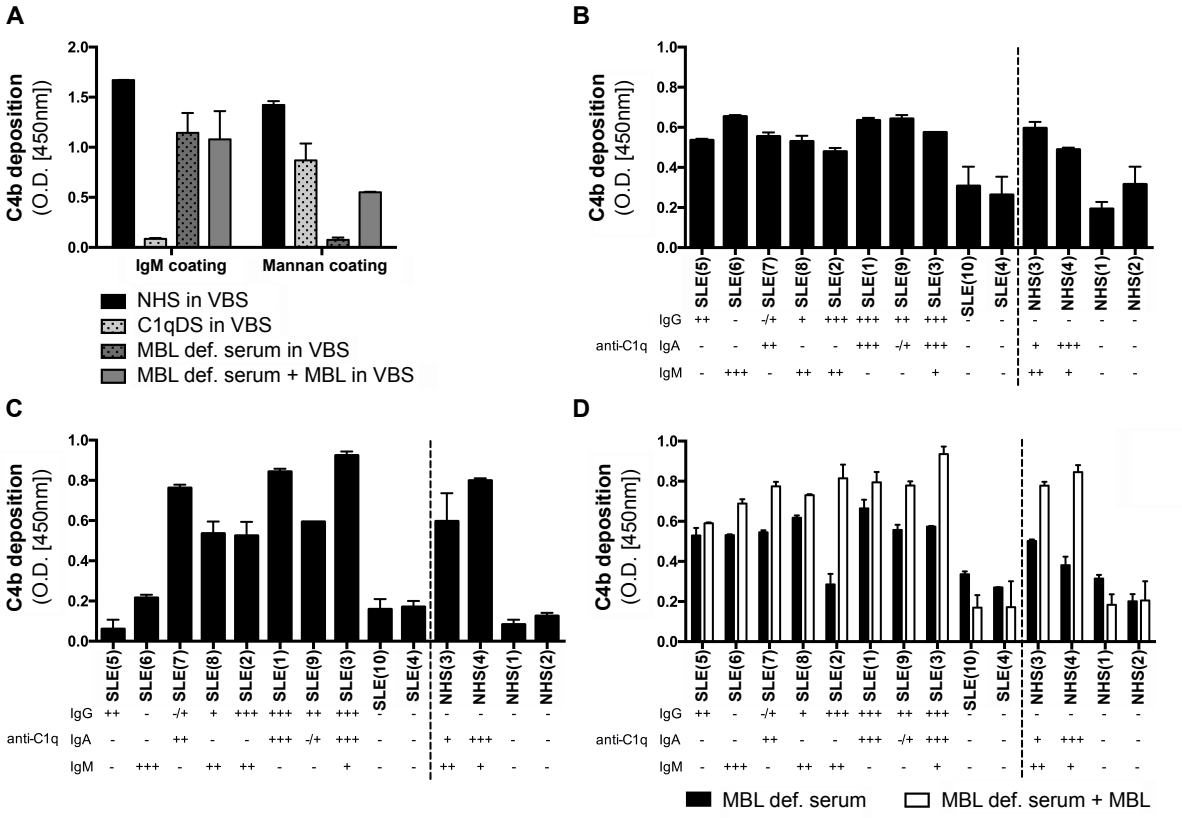


Figure 8: Activation of the CP and the LP mediated by anti-C1q bound to imC1q.

IgM- or mannan-coated plates were incubated with NHS or complement-deficient serum (C1qDS or MBL-deficient serum ± exogenous MBL) and C4b deposition was analyzed (A). As a source of anti-C1q, C1q-coated plates were incubated with heat-inactivated sera of 10 SLE patients (8 anti-C1q positive, 2 anti-C1q negative) or sera of 4 different healthy donors (2 anti-C1q positive, 2 anti-C1q negative) (B-D). As a source of complement NHS (B), C1qDS (C), or MBL-deficient serum with/without 4µg/ml exogenous MBL (D), diluted in VBS, was added to the plates. Finally, C4b deposition was detected. Anti-C1q classes present in the serum samples are indicated for each patient and healthy donor below the figure. Data represent the mean of duplicates ± SEM of one experiment being representative of 2 independent experiments.

Taken together, anti-C1q-dependent complement activation involves both the CP and the LP. Our data indicate that IgG anti-C1q primarily activate the CP and IgA anti-C1q the LP, while IgM anti-C1q seem to be able to activate both, the CP and the LP.

CP activation by anti-C1q dependent on IgG anti-C1q levels

To investigate the potency of anti-C1q to activate the CP of complement, we repeated the *in vitro* assay using purified complement proteins instead of NHS to exclude any contribution of the LP.

As previously, C1q-coated plates were incubated with sera from SLE patients or healthy donors as a source of anti-C1q. Plates were then incubated with purified complement proteins and again C4b deposition was analyzed. Optimization experiments indicated that the complement proteins, C1 and C4, should be incubated one after another, that different incubation times were required for C1 (1h) and C4 (40min), and that at least a molar ratio of 1:2 of C1:C4 was required for a successful anti-C1q-mediated activation (data not shown).

Using serial dilutions of serum from a SLE patient being highly anti-C1q positive or from a healthy donor, we observed dose-dependent complement activation by anti-C1q showing complement activation in lower dilutions as compared to the assay performed with NHS (data not shown).

This assay was repeated with cohorts of SLE and healthy donor sera all being diluted 1:1'000 in high-salt buffer. Under these conditions, anti-C1q from different SLE patients bound to imC1q resulted in the activation of the CP as reflected by C4b deposition in the presence of purified C1 and C4 (Figure 9). C4b deposition triggered by anti-C1q of SLE patients was usually higher as compared to C4b deposition measured with unspecific IgG control coating but lower as compared to IgM control coating (data not shown).

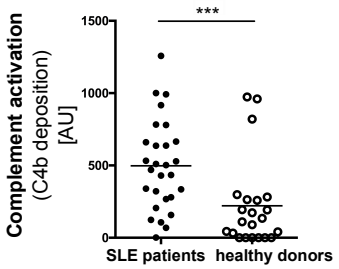


Figure 9: Activation of the CP of the complement system by anti-C1q derived from SLE patients.

To C1q-coated plates, serum of SLE patients (●; n=27) or healthy donors (○; n=22) as a source of anti-C1q was added. After washing the plates, purified C1 (0.5µg/ml) and then C4 (2µg/ml; both diluted in VBS) were added and the activation of the CP was assessed by detecting C4b deposition. C4b deposition values are expressed relative to the same reference serum as described in patients and methods. Samples were tested in duplicates within a single experiment and data points represent the mean of 3 independent experiments. Mann-Whitney U test, $p<0.0005$***.

More importantly, we observed a correlation between IgG anti-C1q binding levels and CP-mediated C4b deposition in SLE patients (Figure 10A). No such correlation was seen when serum of healthy donors was used as a source of anti-C1q (Figure 10B).

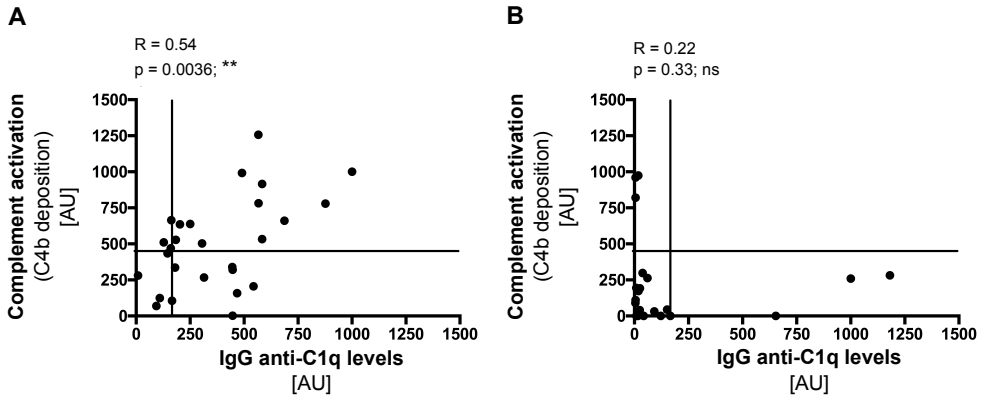


Figure 10: Correlation of IgG anti-C1q levels with the activation of the CP in SLE patients and healthy donors.

The extent of C4b deposition correlated with IgG anti-C1q levels in SLE patients (n=27; (A)) but not in healthy donors (n=22; (B)). Both, IgG anti-C1q levels and C4b deposition values are expressed relative to the same reference serum as described in patients and methods. Crossed horizontal and vertical lines indicate the cut-off values for IgG anti-C1q levels and complement activation. Spearman's rank correlation coefficient (R), $p<0.005$**.

Taken together, the levels of complement activation by SLE anti-C1q correlates with IgG anti-C1q levels, suggesting that anti-C1q exacerbate complement activation.

Discussion

Although it is believed that anti-C1q play a pathogenic role in lupus nephritis, their biological and pathogenic functions are largely unknown. In this context, limited information is available on the complement-activating capacity of anti-C1q. We could now demonstrate that SLE patient-derived anti-C1q activate the complement system *in vitro*. Dependent on the anti-C1q class present in the patient's sera, anti-C1q bound to imC1q activate the CP and the LP. IgG anti-C1q predominantly activate the CP and IgA anti-C1q the LP, whereas IgM anti-C1q seem to trigger both pathways. In the presence of purified complement proteins, we detected specific CP activation correlating with IgG anti-C1q levels of SLE patients. In contrast, anti-C1q did not directly activate the AP.

In principle, it is not surprising that anti-C1q classes activate the complement system by its different pathways given the fact that anti-C1q can be of the IgG-, IgA-, and IgM-class (14-16). It is well known that the different Ig-classes differ in their complement fixing and activating capacity. However, recent publications challenged the traditional view that immunoglobulins mainly activate the complement system via the CP (214, 215). Furthermore, autoantibodies even when belonging to the same Ig-class were shown to have strongly differing complement-activating capacities (206-211).

Our results are not in line with a previous study by Pang et al. (154) showing that affinity purified anti-C1q of lupus nephritis patients inhibit complement activation. The authors hypothesized that this observation could be due to the binding of anti-C1q to the binding site of C1s or C1r inhibiting C1 activation. Nevertheless, anti-C1q are not described to directly bind to C1 (unpublished data; (161, 174)). In addition, Siegert et al. demonstrated *in vivo* and *in vitro* that anti-C1q did not influence C4 consumption concluding that anti-C1q do not interfere with C1 activation by ICs in SLE patients (216). In contrast, we found that anti-C1q bound to imC1q amplify complement activation *in vitro* via the CP and the LP dependent on the anti-C1q Ig-class distribution in the serum of SLE patients. Yet, we did not detect direct AP activation by anti-C1q leading to C3 deposition. Nevertheless, the activation and amplification of the AP have been shown to be of importance in the context of tissue damage in murine models (217). Although, our results do not fully exclude low level direct activation of the AP, we assume that AP activation is rather a secondary event playing an important role as amplifier after initial activation of the complement system via the CP and/or the LP. Indeed, the AP was shown to account for up to 80% of total complement activation, even after initial triggering of the CP or the LP (48).

Our study primarily focused on the analysis of autoantibodies against intact C1q since these autoantibodies have been best described to correlate with SLE disease activity (218, 219). In

addition, using intact C1q maybe best reflect the situation occurring *in vivo*, allowed the analysis of high-affinity antibodies, and avoided manipulations of the molecule as well as the presence of non-physiological components such as peptide 2J (214) and Neutravidine (177).

The main complement activation route in lupus nephritis is considered to be the CP (10, 220). Nevertheless, the LP and the AP seem to also play a role in the progression of this inflammatory kidney disease (221). Data from mouse studies suggest that complement activation in murine lupus nephritis occurs via the CP but also via the LP based on the fact that C3 co-localizes with both C1q and MBL in kidney biopsies (222). Likewise, in the kidneys of lupus nephritis patients immunoglobulins (IgG, IgA, IgM) are deposited close to complement proteins (C1q, MBL, C4, C3) (11, 12). In addition, concentrations of deposited anti-C1q in the glomeruli are up to 50times above those found in the sera of patients (223) leading to the conclusion that anti-C1q deposition and consecutive complement activation in kidneys occur and contribute to the pathogenesis of lupus nephritis. Finally, the link between the CP and anti-C1q is underlined by the negative correlation of anti-C1q titers with C1q serum levels and other complement components (29). Furthermore, deposition of C1q and nucleosomes to glomerular endothelial cells occurs *in vitro* and the subsequent binding of different autoantibodies, including anti-DNA and anti-C1q, leads to complement activation (224).

With regard to downstream mechanisms, anti-C1q are believed to involve both, activation of the complement system as well as recruitment of Fc receptors upon binding to e.g., C1q-containing ICs deposited in the kidneys with consecutive amplification of the inflammatory process during flares (10). Anti-C1q were described to bind to C1q-containing ICs deposited in mouse glomeruli (225) leading to the hypothesis that C1q bound to ICs deposited in the kidneys serves as a target for anti-C1q.

Next to its involvement in host defense, the complement system has multiple other functions such as bridging innate with adaptive immunity and clearing apoptotic cell material. The efficient and fast clearance of early apoptotic cell material is essential to avoid inflammatory and autoimmune processes (48, 226). The structurally similar molecules, C1q and MBL, both bind to the surface of apoptotic cells, facilitate their uptake by phagocytes (23, 24), and trigger the activation of the CP and the LP which then in turn can activate the AP (22, 77, 227).

During a flare even more cells in the kidneys might render apoptotic, as occurring in the glomeruli of C1q-deficient mice and highlighting the importance of C1q in the removal of apoptotic cell material (5). In this context, the binding of anti-C1q to C1q might interfere with biological functions of C1q beyond complement activation, e.g., the clearance of apoptotic cell material. As has been shown previously, C1q bound to the surface of apoptotic cells is

targeted by anti-C1q (7), which thereby decreases phagocytosis of apoptotic cells by THP-1 cells (154). Whether anti-C1q bound to C1q on apoptotic cells also amplify complement activation and as a consequence alter the interaction with immune cells, needs to be further studied.

Anti-C1q have not only been described in SLE patients, but also occur in other autoimmune and renal diseases such as RA, hypocomplementemic urticarial vasculitis, acute post-streptococcal glomerulonephritis, and IgA nephropathy (14, 228). Whereas anti-C1q seem to have a pathogenic role in severe lupus nephritis, in RA patients the occurrence of anti-C1q is not more frequent than in healthy individuals. Nevertheless, the prevalence of anti-C1q is increased in patients having severe RA accompanied by rheumatoid vasculitis, with IgA anti-C1q being the predominant Ig-class (14). Although the pathogenic role of IgA anti-C1q in RA needs to be investigated, our data suggest that IgA anti-C1q might contribute to the pathogenesis and tissue injury in severe RA by triggering complement activation via the LP.

In conclusion, we can show that both the CP and the LP play a critical role in complement activation triggered by anti-C1q. Our results provide new insights into the pathogenic mechanisms of anti-C1q and their role in lupus nephritis. These new insights eventually might help in the development of complement-specific treatments (229).

Part 2

Anti-C1q autoantibodies from systemic lupus erythematosus patients induce a proinflammatory phenotype in macrophages

Abstract

Autoantibodies against C1q (anti-C1q) are frequently found in patients with systemic lupus erythematosus (SLE) and correlate with the occurrence of proliferative lupus nephritis. A previous study of anti-C1q in experimental lupus nephritis demonstrated an important role of Fcgamma receptors (FcγR) in the pathogenesis of lupus nephritis suggesting a direct effect on phagocytes. Therefore, we developed an *in vitro* model to study the effect of SLE patient-derived anti-C1q bound to immobilized C1q (imC1q) on human monocyte-derived macrophages (HMDMs) obtained from healthy donors and SLE patients. HMDMs were investigated by analyzing the cell morphology, LPS-induced cytokine profile, surface marker expression, and the phagocytosis rate of apoptotic Jurkat cells. Morphologically, bound anti-C1q induced cell aggregations of HMDMs when compared to imC1q or IgG alone. In addition, anti-C1q reversed the effect of imC1q alone shifting the LPS-induced cytokine release towards a proinflammatory response. By FcγR-blocking experiments, the secretion of proinflammatory cytokines was found to be mediated via FcγRII. The anti-C1q-induced inflammatory cytokine profile was accompanied by a downregulation of CD163 and an upregulation of LPS-induced CD80, CD274, and MHC class II. Finally, HMDMs primed on bound anti-C1q versus imC1q alone displayed a significantly lower phagocytosis rate of early and late apoptotic cells accompanied by a reduced MerTK expression. Interestingly, anti-C1q-dependent secretion of proinflammatory cytokines was similar in SLE patient-derived cells with the exception of IL-10 being slightly increased. In conclusion, anti-C1q induced a proinflammatory phenotype in HMDMs reversing the effects of imC1q alone. This effect might exacerbate underlying pathogenic mechanisms in lupus nephritis.

Introduction

Systemic lupus erythematosus (SLE) is characterized by B cell hyperactivity, a variety of antibodies directed against autoantigens, such as intra-cellular components or plasma proteins, the formation of immune complexes (ICs), and aberrant complement activation (44, 45).

Inherited as well as acquired complement deficiencies have been associated with the development and pathogenesis of SLE. Particularly, primary deficiencies of early components of the classical pathway (CP) (C1q, C4, C2) are strongly linked to SLE. The strongest genetical susceptibility to develop SLE is homozygous C1q deficiency, underlining that complement plays a major role in the pathogenesis of SLE (3). However, most SLE patients do not suffer from primary C1q deficiency, but in general, ongoing complement activation is accounted for low or undetectable complement levels. A rational reason for low C1q levels are autoantibodies against C1q (anti-C1q) which are present in 20 to 50% of unselected SLE patients and their occurrence correlates with both, low complement levels as well as lupus nephritis (7-9). Even though, these autoantibodies are linked to renal involvement, the direct evidence how these autoantibodies contribute to the pathogenesis of lupus nephritis is not yet available. Animal models suggested that renal inflammation is only induced by anti-C1q in combination with preformed glomerular C1q-containing ICs, requiring both complement activation as well as Fcgamma receptor (FcγR) triggering (10). Recently, Pang et al. demonstrated that affinity purified anti-C1q from lupus patients bind to C1q on early apoptotic cells and thereby decrease the uptake of apoptotic cells by THP-1 cells (154). However, the direct downstream effect of anti-C1q on professional phagocytes is not well understood.

C1q, the recognition molecule of the C1 complex, acts as the initiator of the CP of the complement system (1). Beyond complement activation, C1q serves as a regulatory protein during inflammatory processes including autoimmunity. The regulatory functions also affect cell differentiation, chemotaxis, migration, and survival (84-87). Moreover, C1q participates in the clearance of apoptotic cell material. C1q can directly bind to the surface of apoptotic cells via its globular heads (76, 78). The collagen-like tails then interact with phagocytes via C1q receptors (22), thereby facilitating the engulfment of apoptotic cells (23, 120). Additionally, studies from different research groups demonstrated that C1q not only enhances the uptake of apoptotic cell material, but also modifies the cytokines profile released by phagocytic cells towards a less inflammatory response during phagocytosis (23, 120, 135, 136). During the uptake of early and late apoptotic cells, C1q exerted a potent inhibitory capacity in macrophage-mediated inflammation (25). These data suggest that C1q is crucial in limiting

inflammation during the uptake of apoptotic cells. Furthermore, C1q-polarization of macrophages might induce an anti-inflammatory (M2-like) phenotype.

Macrophages play an important role in host defense, inflammatory processes, tissue remodeling and homeostasis. The functional profile of macrophages is determined by their activation and exposure to environmental factors such as cytokines and growth factors during their differentiation from monocytes into macrophages (185). *In vitro*, monocytes can be polarized into different macrophage subtypes by specific cytokines. Based on the stimuli providing an activation signal, macrophages have been divided in a continuum between 2 functionally polarized states: proinflammatory macrophages (termed M1) and anti-inflammatory macrophages (termed M2). The polarized macrophages exhibit functional differences as evident by their phenotypic profiles such as cytokine release and surface markers (185-187). M1 macrophages mainly produce proinflammatory cytokines, phagocyte microorganisms, and are often linked to tissue injury and inflammation, whereas M2 macrophages display in general a low antigen-presenting capacity, inhibit and prevent T cell activation, and are associated with tissue repair and fibrosis (188, 189). However, polarization of macrophages is not permanent, but rather milieu dependent and reversible (192).

Functional defects in the cells of the monocyte-macrophage lineage from SLE patients are well known, although the underlying molecular mechanism is not fully understood. SLE patient-derived macrophages exhibit a defect in the phagocytosis of apoptotic cell material correlating with low complement levels (17, 18). Additionally, monocytes from SLE patients have an abnormal cytokine secretion profile in response to apoptotic cells independent of the monocyte's phagocytic efficiency or the patient's disease state (19). These reports underline that phagocytes and complement play a key role in the pathogenesis of lupus.

The aim of the study was to determine the simultaneous interaction of human monocyte-derived macrophages (HMDMs) with bound autoantibodies, on the one hand, and the immuno-regulatory C1q molecule, on the other hand, using SLE patient-derived high-affinity anti-C1q which have been found to correlate with disease activity.

Patients and methods

Anti-C1q source/IgG source

A cohort of 17 SLE patients (Table 8) and 15 healthy control donors was included in the study.

Table 8: Characterization of SLE patients used as a source for anti-C1q.

Pat ID (sex (f/m))	**Age** (y)	**LN** (yes/no) [1,2]	**Anti-C1q levels** [AU] (cut-off: 98AU)	**C1q levels** [µg/ml]	**Hypocomplementemia** (low C3 and C4) (yes/no) [1]	**CP activation** (yes/no)	**ANA/ Anti-dsDNA** (yes/no)	**Medication**
SLE(1) (f)	28	yes (IV)	1'000	0	yes	yes	yes	AZA, CTX, Pred, RIT
SLE(2) (f)	36	yes (III)	625	0	yes	yes	yes	AZA, CTX, Pred
SLE(3) (f)	25	yes (IV)	533	0	yes	yes	yes	AZA, Pred
SLE(4) (f)	27	yes (IV)	782	15.7	yes	yes	yes	MMF
SLE(5) (f)	31	yes (IV)	494	95.9	yes	yes	only ANA	none
SLE(6) (m)	45	yes (IV)	233	13.8	yes	yes	yes	none
SLE(7) (m)	56	yes (II)	396	54.8	yes	yes	yes	Pred
SLE(8) (f)	50	yes (III)	543	0	yes	yes	yes	HCQ, Pred
SLE(9) (f)	29	yes (IV)	584	19.9	yes	yes	yes	MTX, Pred
SLE(10) (m)	45	yes (III)	268	122	yes	yes	yes	Pred
SLE(11) (f)	50	yes (II)	131	74.7	no (borderline)	no	yes	none
SLE(12) (f)	53	yes (II)	180	82.6	only low C4	yes	yes	none
SLE(13) (f)	57	R	16	79.9	no	no	yes	none
SLE(14) (f)	55	R	49	98.4	no	no	only ANA	none
SLE(15) (m)	40	R	72	73.0	no	no	yes	AZA, HCQ, Pred
SLE(16) (f)	34	R	14	105.3	no	no	only ANA	MMF
SLE(17) (f)	37	R	57	86.6	no	no	only ANA	none

Legend: AU: arbitrary units; AZA: Azathioprine; CP: classical pathway; CTX: Cyclophosphamide; f: female; HCQ: Hydroxychloroquine; LN: lupus nephritis; m: male; MMF: Mycophenolate mofetile; MTX: Methotrexate; Pred: Prednisone; R: remission; RIT: Rituximab; y: years

[1] Information at time point of blood sampling

[2] Classification of lupus nephritis according to WHO classification

All SLE patients fulfilled at least 4/11 criteria of the American College of Rheumatology (212, 213). Collection and use of serum samples were approved by the local Ethics Committee (EKZ-No.: 110/04; 130/05).

Isolation and differentiation of HMDMs

HMDMs were derived from CD14-positive monocytes isolated from fresh buffy coat's (Blood Transfusion Centre, Basel, Switzerland). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation (Histopaque 1077, Sigma-Aldrich, MO, USA). CD14-positive monocytes were isolated from PBMCs using CD14 Microbeads (MACS, Bergisch Gladbach, Miltenyi Biotec, Germany) according to the manufacturer's instructions (the average purity of the CD14-positive monocyte fraction was always >95-98% as assessed by flow cytometry). HMDMs were generated from CD14-positive monocytes by culture in Dulbecco modified essential medium supplemented with 1% penicillin/streptomycin (DMEM+; both obtained from GIBCO, Invitrogen, UK) and 10% NHS (pooled from 40 healthy donors). The culture was maintained in 5% CO_2 at 37°C for 7 days and the media were exchanged every 2-3 days.

SLE patient-derived macrophages

15ml of heparinized venous blood was obtained from SLE patients (Table 9) after written consent according to the local Ethics committee (EKZ-No.: 2014/125).

Table 9: Characterization of SLE patients from whom CD14-positive monocytes were isolated[1].

Pat ID (sex (f/m))	**Age** (y)	**Disease duration** (y)	**Anti-C1q levels** (cut-off: 98AU)	**C1q levels** [µg/ml]	**Disease state**	**Medication**
SLE(A) (f)	66	0	342	49.7	flare (stroke; LN)	Pred
SLE(B) (f)	31	12	203	40.0	R (LN (class IV))	AZA, Pred, Rit
SLE(C) (m)	30	7	92	152	R	HCQ
SLE(D) (f)	48	20	51	108.3	R (LN (class IV))	MMF, Pred
SLE(E) (f)	34	0.5	1'080	0	R (no LN)	MMF
SLE(F) (m)	40	14	73	94.0	R	Pred

Legend: AU: arbitrary units; AZA: Azathioprine; f: female; HCQ: Hydroxychloroquine; LN: lupus nephritis; m: male; MMF: Mycophenolate mofetile; Pred: Prednisone; R: remission; Rit: Rituximab; y: years;
[1] Information at time point of blood sampling

All SLE patients fulfilled at least 4/11 criteria of the American College of Rheumatology (212, 213). CD14-positive monocytes were isolated and differentiated as described above with one

exception. Adherent monocytes were differentiated into macrophages in DMEM+ supplemented with 10% autologous serum for 7days. For the analysis of LPS-induced cytokine release, the patient's differentiated macrophages were stimulated as described below. For the stimulation, the patient's own anti-C1q were also included in the experimental setting.

In vitro model used for the stimulation of HMDMs

After 7 days, HMDMs were harvested using ice-cold PBS (GIBCO, Invitrogen, UK), resuspended in DMEM+ supplemented with 0.1% human serum albumin (HSA; HSA-DMEM; Sigma-Aldrich, MO, USA) at $0.5x10^6$cells/ml, and used for stimulation experiments. Therefore, 96well plates (MaxiSorp, Nalge Nunc International, Denmark) were coated with purified C1q (immobilized C1q (imC1q); Complement Technology, TX, USA) or HSA at 5µg/ml in coating buffer (0.1M sodium carbonate buffer, pH 9.6) overnight at 4°C. The plates were washed twice with PBS and C1q-coated wells were incubated with 100µg/ml purified total IgG (purified by ProteinG affinity columns) from SLE patients or healthy donors diluted in high-salt buffer (PBS/1M NaCl) for 1h at 37°C. Before use, each IgG preparation was centrifuged at 14'000xg for 30min at 4°C. After washing the plates 4times with PBS, HMDMs (100µl of $0.5x10^6$cells/ml: 50'000cells/well) were added to the wells, cells were allowed to adhere for 60min at 37°C, and where indicated 10ng/ml lipopolysaccharide (LPS; *E. coli*: O127:B8; Sigma-Aldrich, MO, USA) or DMEM-HSA was added, and cells were incubated for 18h.

For FcγR-blocking experiments, FcγRII were blocked by incubating HMDMs with 8µg/ml anti-FcγRII (anti-human CD32 $F(ab')_2$, clone: 7.3; Ancell Corporation, MN, USA) for 30min at 4°C in DMEM-HSA before proceeding with stimulation experiments as described above.

Morphology of HMDMs

The morphology of HMDMs was assessed using the above described *in vitro* model and an Olympus IX50 inverted phase contrast microscope (Olympus, Hamburg, Germany) with magnifications of 10x and 20x. The size of cell aggregations was quantified using ImageJ software (ImageJ software 1.47, USA). Data are expressed relative to the cell size formed by HMDMs incubated on imC1q alone which was set to 1.

Quantification of LPS-induced cytokine release

After the stimulation of HMDMs on different coatings for 18h, supernatants (SN) were collected, centrifuged to remove cellular debris, and kept at -80°C until analysis. The concentrations of IL-1β, IL-6, TNFα, and IL-10 were measured using Opt ELISA kits (BD

Biosciences, CA, USA) according to the manufacturer's instructions. All samples were analyzed in duplicates.

Expression of surface markers

After stimulating HMDMs on different coatings for 18h using the *in vitro* model described above, cells were collected and washed twice with ice-cold FACS buffer (PBS/1% BSA (Sigma-Aldrich, MO, USA)/1mM sodium azide). Cells were resuspended in FACS buffer at $1x10^6$cells/100µl. Non-specific binding to FcγRs was blocked by incubating cells with 2mg/ml human IgG/$1x10^6$ cells for 45min at 4°C. For FACS analysis of surface markers, the following mouse mABs conjugated with FITC were used: CD14 (Immunotools, Friesoythe, Germany), CD80 and CD86 (both obtained from BD Biosciences, CA, USA), CD206 (Biolegend, Fell, Germany), and MHC class II (Immunotools, Friesoythe, Germany), mouse mABs conjugated with PE: CD273 and CD274 (both obtained from Biolegend, Fell, Germany), and Mer tyrosine kinase (MerTK; R&D systems, MN, USA), or mouse mAB conjugated with APC: CD163 (Biolegend, Fell, Germany). In each experiment, parallel stainings with appropriate isotype-matched controls IgG1-FITC, IgG2a-FITC, IgG1-APC (all obtained from Immunotools, Friesoythe, Germany), or IgG2b-PE (Biolegend, Fell, Germany) were performed. For stainings, cells were resuspended at $5x10^5$cells/100µl in FACS buffer and stained with FITC-, PE-, or APC-conjugated antibodies or their matched isotype-control for 45min at 4°C. Cells were washed twice with FACS buffer and resuspended in FACS buffer. For each dataset, 10'000 events in the viable cell gate (Propidium iodide (PI; Sigma-Aldrich, MO, USA) exclusion of dead cells) were acquired using a FACSAccuri (BD Biosciences, CA, USA) and analyzed using Flowjow software (Tree Star Inc., OR, USA). The final geometric mean fluorescence intensities (gMFI) were calculated by subtracting the gMFI of the corresponding isotype control from the gMFI of the sample.

Phagocytosis assays

Endocytic activity of stimulated HMDMs

The endocytic activity of stimulated HMDMs was measured by analyzing the uptake of FITC-conjugated dextran (molecular mass: 40'000kDa; Sigma-Aldrich, MO, USA). $5x10^5$cells/100µl stimulated HMDMs were incubated with 0.5mg/ml FITC-conjugated dextran in media for 0, 30, or 60min at 37°C or 4°C to measure specific uptake versus non-specific binding, respectively. HMDMs were washed 3times with FACS buffer and the uptake of FITC-conjugated dextran was analyzed by flow cytometry using a FACSAccuri.

Phagocytosis of apoptotic cells by stimulated HMDMs

Jurkat T cells (Jurkats) were cultured in RPMI 1640 supplemented with 1% penicillin/streptomycin, 1% L-glutamine, 26mM Hepes, and 10% FCS (complete RPMI; all reagents obtained from GIBCO, Invitrogen, UK). Apoptosis was induced by UV-light treatment (Stratalinker 1800, Stratagene) at 254nm for 1 min at a cell concentration of $2x10^6$cells/ml. Irradiated Jurkats were further cultured for 16h in complete RPMI to obtain early apoptotic cells or in FCS free complete RPMI to obtain late apoptotic cells. Prior to apoptosis induction, Jurkats were fluorescently labeled with 5µM CFSE (Invitrogen, Molecular Probes, UK) according to the manufacturer's instructions. Apoptotic cells were characterized by double-staining for AnnexinV (AnV; Immunotools, Friesoythe, Germany) and PI. For phagocytosis assays, HMDMs were primed for 18h as described above, harvested, and washed twice with DMEM+. CFSE-labeled apoptotic Jurkats were coincubated with differently primed HMDMs (10^5 cells) at a 1:1 ration for 0 or 30min at 37°C or 4°C in a total volume of 200µl phagocytosis buffer (DMEM+/26mM Hepes/5mM $MgCl_2$). Phagocytosis was stopped by adding cold FACS buffer, and unphagocytosed Jurkats were washed away. HMDMs were further stained with an APC-conjugated mAB against CD14 (Immunotools, Friesoythe, Germany) and the uptake of apoptotic Jurkats was analyzed by flow cytometry. The percentage of $CD14^+CFSE^+$ double-positive cells was used to assess the percentage of HMDMs that phagocytosed (incubated at 37°C) and/or bound (incubated at 4°C) apoptotic Jurkats. The uptake of apoptotic Jurkats by HMDMs was expressed as percent phagocytosis (phagocytosis [%]) defined as $CD14^+CFSE^+$ double-positive cells divided by the total of $CD14^+$-positive cells multiplied by 100.

Statistical analysis

Data are expressed as mean ± SEM if not stated otherwise. Statistical analyses were performed using the Wilcoxon-matched pair test or the Mann-Whitney U test to compare 2 groups. To compare more than 2 groups, one-way ANOVA followed by Bonferroni's post-test was used as indicated. Data were analyzed using GraphPad Prism software (GraphPad Prism, CA, USA). A $p<0.05$ was considered statistically significant.

Results

ImC1q and anti-C1q bound to imC1q modify the morphology of HMDMs

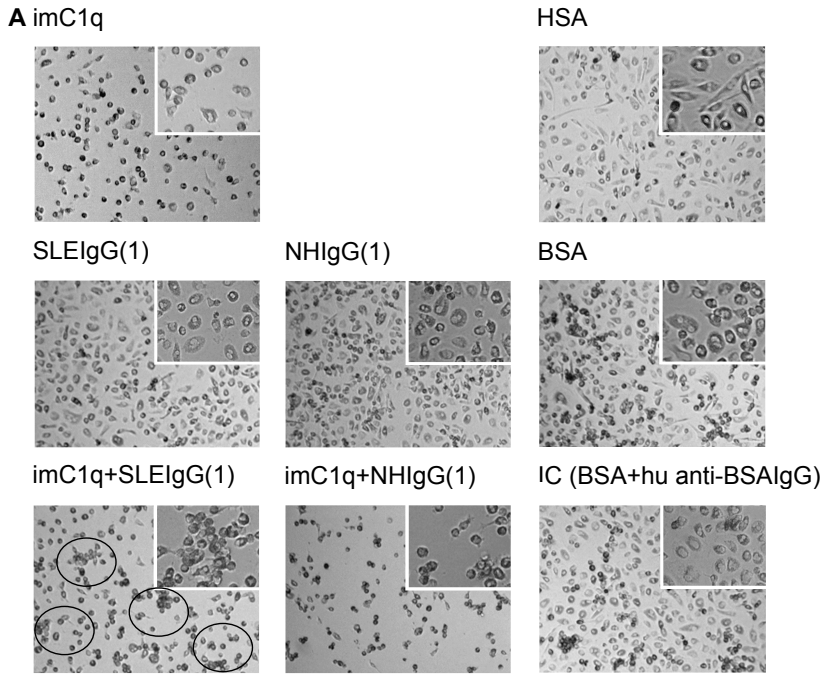
CD14-positive monocytes were cultured for 7 days to obtain HMDMs. Cells were then harvested and reseeded on wells with different coatings. The cell morphology on these coatings was assessed using phase contrast microscopy.

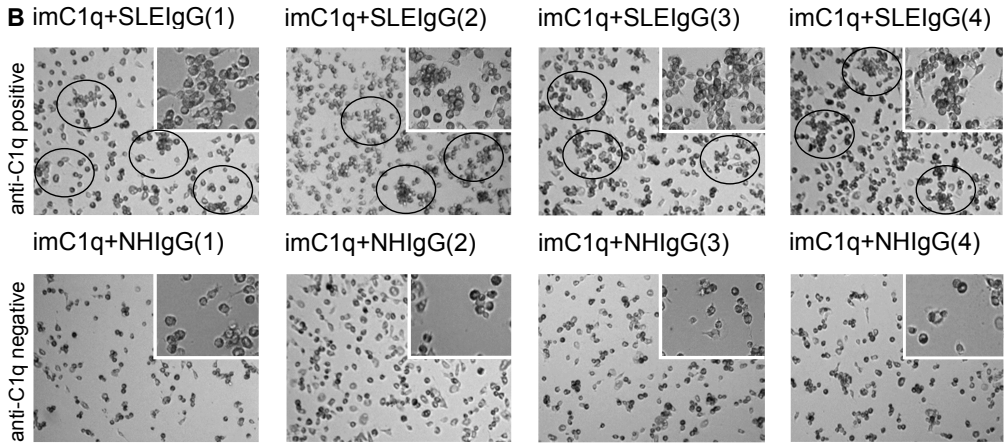
HMDMs stimulated for 18h on HSA or imC1q showed different morphologies (Figure 11A). Cells on imC1q exhibited a circular cell shape as compared to those plated on HSA, which had a more elongated and spindle-like cell shape. This observation is consistent with previous findings on changes in the morphology of monocytes plated on imC1q as compared to those on HSA (135).

Intriguingly, when HMDMs were plated on anti-C1q bound to imC1q (imC1q+SLEIgG) the cells still had a circular cell shape, but additionally formed cell aggregates (Figure 11A). This effect was anti-C1q specific, since HMDMs plated on bound anti-C1q derived from anti-C1q positive healthy donors also resulted in aggregations (data not shown). However, no cell aggregations could be observed when using ICs (BSA-human IgG anti-BSA) (Figure 11A). Furthermore, HMDMs plated on coated purified IgG also did not lead to cell aggregates, neither on SLEIgG nor on NHIgG (Figure 11A). The formation of cell aggregates was not due to increased cell death since double-staining for both, AnV and PI, did not show any significant difference between the different priming conditions (data not shown).

Comparing total IgG derived from 4 different SLE patients to IgG from 4 healthy donors confirmed that the formation of cell aggregates by HMDMs on SLE patient's anti-C1q can be observed in several patient's anti-C1q but not in anti-C1q negative healthy donors (imC1q+NHIgG) (Figure 11B).

Next, we studied the effects of anti-C1q from a whole cohort of SLE patients with variable anti-C1q titers and healthy donors (Figure 11C). The average size of cell aggregates formed by HMDMs on bound anti-C1q was 1.8-fold increased as compared to the one of HMDMs plated on imC1q alone or on imC1q+NHIgG (imC1q+SLEIgG: 1.79±0.0.150 vs imC1q+NHIgG: 1.17±0.082; p=0.0037**).


A imC1q
HSA
SLEIgG(1)
NHIgG(1)
BSA
imC1q+SLEIgG(1)
imC1q+NHIgG(1)
IC (BSA+hu anti-BSAIgG)


B imC1q+SLEIgG(1)
imC1q+SLEIgG(2)
imC1q+SLEIgG(3)
imC1q+SLEIgG(4)
anti-C1q positive
imC1q+NHIgG(1)
imC1q+NHIgG(2)
imC1q+NHIgG(3)
imC1q+NHIgG(4)
anti-C1q negative

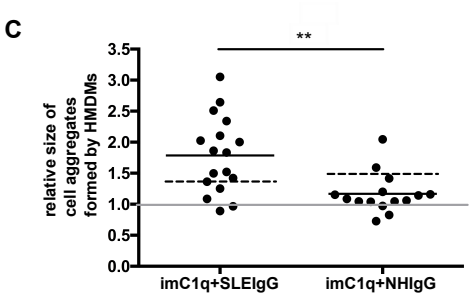

C
**
relative size of cell aggregates formed by HMDMs
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
imC1q+SLEIgG
imC1q+NHIgG

Figure 11: ImC1q and anti-C1q bound to imC1q affect the morphology of HMDMs.

HMDMs were incubated on different coatings (as indicated in the figure) for 18h and their morphology was analyzed using an Olympus phase contrast microscope (original magnification, x10/x20). HMDMs were incubated on HSA (5µg/ml) or imC1q (5µg/ml), coated purified total IgG (50µg/ml) obtained from an anti-C1q positive SLE patient (SLEIgG(1)) or from a healthy donor (NHIgG(1)), on ICs (BSA–human anti-BSA IgG) or BSA only (A). HMDMs were incubated on imC1q+SLEIgG from 4 different SLE patients (all anti-C1q positive) or on imC1q+NHIgG from 4 healthy control donors (all anti-C1q negative) (B). Shown are the results of one donor used to obtain HMDMs representative for 5 independent experiments. HMDMs were incubated on IgG derived from a cohort of anti-C1q positive and negative SLE patients (n=17) and healthy donors (n=15) (C). The size of cell aggregates formed by HMDMs was analyzed using ImageJ software. Data are expressed relative to the cell size formed by HMDMs incubated on imC1q only (grey line; relative size=1). Each data point represents pooled mean values of 5 different experiments. Data points above the dashed lines indicate anti-C1q positive SLE patients and healthy donors. Mann-Whitney U test, $p<0.005$**.

Anti-C1q induce a proinflammatory cytokine response in HMDMs

Beyond complement activation, C1q has been described to act as an anti-inflammatory regulator in immune cell processes. For example, C1q induces a less inflammatory response in phagocytes in combination with a proinflammatory stimulus such as LPS regarding cytokine secretion and cytokine mRNA levels (25, 120).

Therefore, we next assessed whether anti-C1q bound to imC1q change the LPS-induced cytokine profile secreted by HMDMs as compared to imC1q or HSA alone. For this purpose, HMDMs were incubated on HSA, imC1q, or imC1q with different anti-C1q positive SLEIgG or anti-C1q negative NHIgG in the presence or absence of the TLR4-ligand LPS for 18h (Figure 12).

HMDMs mostly did not produce detectable cytokine levels in the absence of LPS independent of the coatings (data not shown).

However, in accordance with previous reports (25, 120), imC1q significantly downregulated LPS-induced secretion of proinflammatory cytokines, such as IL-1β, IL-6, and TNFα, as compared to HSA alone (IL-1β/IL-6: p=0.031*; TNFα: p=0.0435*) (Figure 12A-C; first horizontal panel). The suppressive effect of imC1q alone was reversed by SLE patient-derived anti-C1q bound to imC1q as evident by a significant upregulation of proinflammatory cytokine levels as compared to imC1q+NHIgG (IL-1β/TNFα: p=0.0001***; IL-6: p=0.0003***) (Figure 12A-C; second horizontal panel). Induction of a proinflammatory cytokine response could also be observed when NHIgG from anti-C1q positive healthy donors were used (Figure 12A-C; second horizontal panel, indicated by black arrows).

In contrast, imC1q upregulated the LPS-induced level of the anti-inflammatory cytokine IL-10 as compared to HSA-coating (IL-10: p=0.02*) (Figure 12D; first horizontal panel). Interestingly,

SLE patient-derived anti-C1q significantly attenuated LPS-induced IL-10 production as compared to imC1q+NHIgG (IL-10: p=0.0354*) (Figure 12D; second horizontal panel). Again, healthy donor-derived anti-C1q induced a similar effect as anti-C1q from SLE patients did (Figure 12D; second horizontal panel, indicated by black arrows).

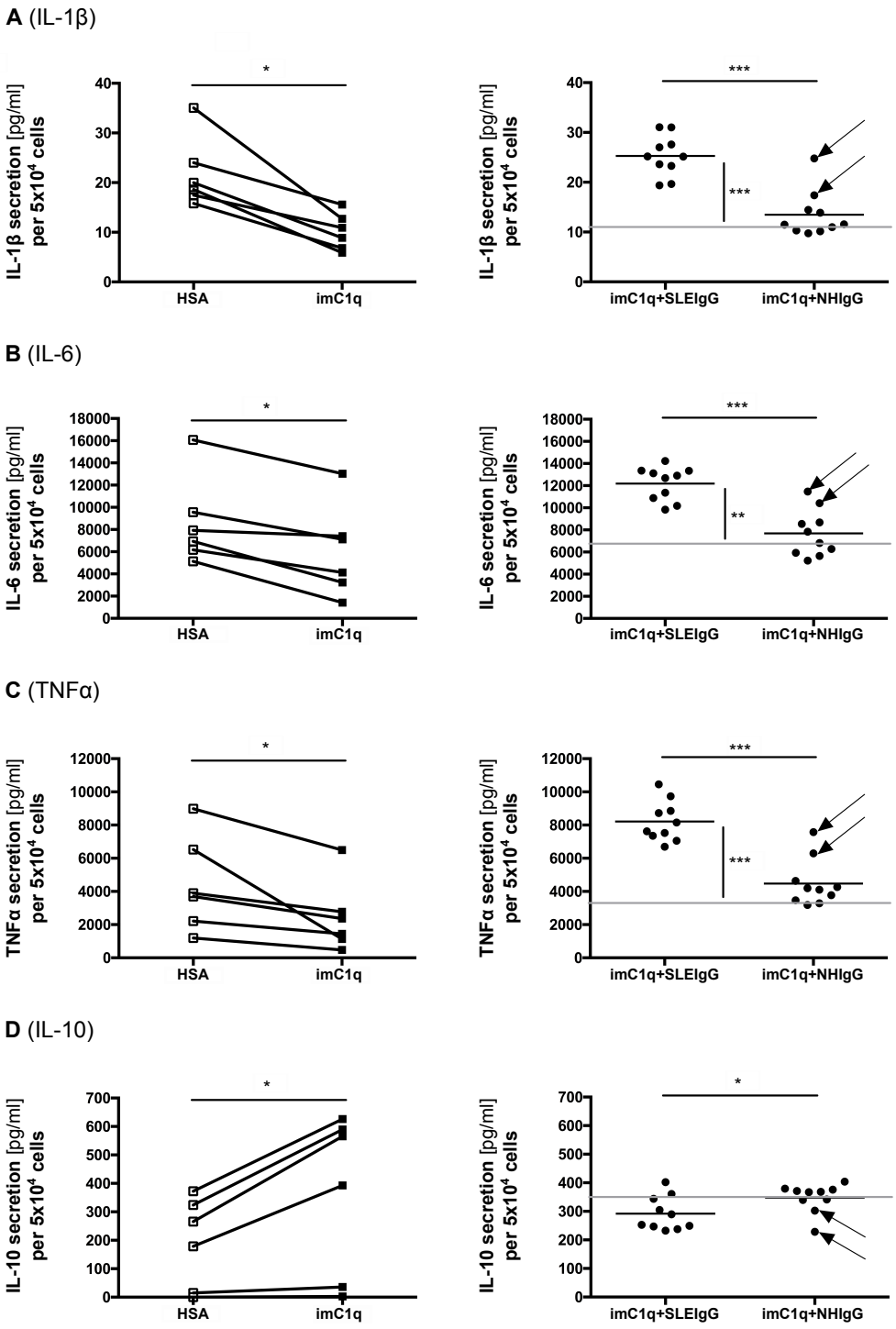


Figure 12: Anti-C1q bound to imC1q shift LPS-induced cytokine secretion by HMDMs towards a proinflammatory response.

After stimulation of HMDMs for 18h on different coatings, LPS-induced concentrations of secreted cytokines (IL-1β (A), IL-6 (B), TNFα (C), and IL-10 (D)) in SN were measured by ELISA. Data sets on the first horizontal panel show secreted LPS-induced cytokine levels of HMDMs incubated on HSA (□) or imC1q (■). Data represent cytokine release levels of 6 unrelated healthy donors used to obtain HMDMs. Wilcoxon-matched pair test, $p<0.05$*. Data sets on the second horizontal panel display LPS-induced cytokine levels released by HMDMs when adhered to imC1q+SLEIgG or imC1q+NHIgG (n=10 different IgG preparations each). Each data point represents pooled cytokine levels (mean) of 6 independent experiments. Grey lines represent mean cytokine levels secreted by HMDMs incubated on imC1q alone. Black arrows indicate anti-C1q positive healthy donors. Mann-Whitney U test, $p<0.05$*, $p<0.005$**, $p<0.0005$***.

When SLEIgG of anti-C1q negative patients were incubated on imC1q, no differences to NHIgG were detectable for any of the tested cytokines (data not shown). In addition, we found that IgG anti-C1q levels of SLE patients correlated with the LPS-induced secretion of all cytokines tested (data not shown).

Taken together, imC1q-bound autoantibodies shifted the LPS-induced cytokine levels towards an inflammatory response, as evident by an upregulation of IL-1β, IL-6, and TNFα secretion, and accompanied by a slight downregulation of IL-10 secretion as compared to imC1q with control NHIgG.

Anti-C1q-triggered proinflammatory cytokine response is mediated by a FcγRII-dependent pathway

Several reports suggest that FcγR-engagement by deposited ICs in kidneys is crucial for the development of lupus nephritis (10, 230). Given the close correlation of lupus nephritis and anti-C1q, we hypothesized that FcγR are of crucial importance for the proinflammatory effects of anti-C1q as described before. Since IgG2 is the predominant anti-C1q class and can trigger CD32 (FcγRII) (167, 181), we preincubated HMDMs in the presence or absence of a CD32-blocking antibody. Next, HMDMs were incubated as outlined above and LPS-induced cytokine levels were analyzed (Figure 13). Blocking of FcγRII led to a decreased secretion of all proinflammatory cytokines tested down to levels as observed in the absence of anti-C1q (IL-1β: 84.3±13.5%, $p=0.021$**; IL-6: 63.1±9.8%, $p=0.0003$***; TNFα: 86.0±5.3%, $p=0.0011$**) (Figure 13A-C). In contrast, IL-10 secretion was not significantly altered by CD32-blocking (IL-10: -22.2±7.482.0%, $p=0.075$, ns) (Figure 13D).

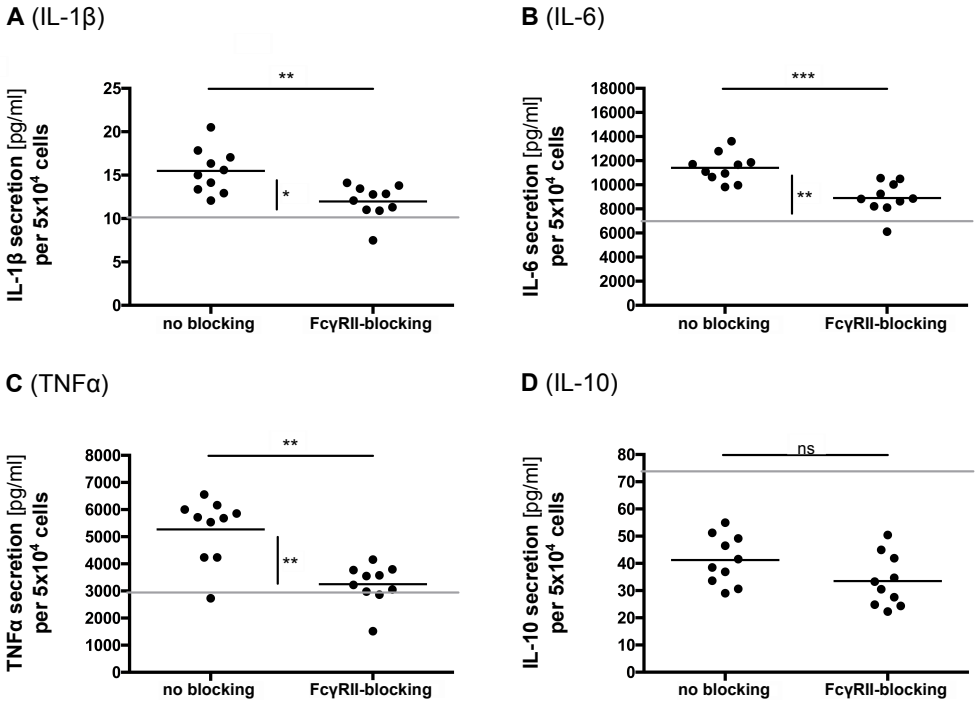


Figure 13: LPS-induced secretion of proinflammatory cytokines by anti-C1q is FcγRII-mediated.

HMDMs were preincubated in the absence or presence of CD32-blocking AB (8µg/ml) and further incubated on imC1q+SLEIgG (n=10). After 18h of stimulation, LPS-induced IL-1β (A), IL-6 (B), TNFα (C), and IL-10 (D) levels were measured. Each data point represents a mean value of 6 independent experiments using 6 different HMDMs preparations. Grey lines show mean cytokine levels secreted by HMDMs incubated on imC1q alone. Mann-Whitney U test, $p<0.05$*, $p<0.005$**, $p<0.0001$***.

Anti-C1q induce a mixed phenotype in HMDMs

Since the effect of anti-C1q can be found in the induction of a proinflammatory and activating response as evident by both, morphological changes and LPS-induced cytokine secretion levels, we next assessed whether anti-C1q also have an effect on surface markers expressed by HMDMs. For this, we evaluated the expression of several surface markers including CD14, co-stimulatory receptors (CD80, CD86, CD273, CD274), mannose receptor (CD206), scavenger receptor (CD163), and MHC class II (Figure 14).

Fully differentiated HMDMs were primed as described before and then stained for surface markers. As control for M2 macrophages untreated HMDMs and for M1 macrophages HMDMs stimulated with LPS were used.

HMDMs incubated on imC1q significantly upregulated CD14 and CD163 as compared to untreated control cells (CD14: p=0.011**; CD163: p=0.048*), whereas CD86 and CD274 were downregulated (CD86: p=0.0036**; CD274: p=0.048*). HMDMs primed on anti-C1q bound to imC1q showed a trend to upregulate MHC class II as compared to imC1q alone (MHC class II:

p=0.0625, ns). On the contrary, expression of CD163 was reversed by HMDMs stimulated on bound anti-C1q as compared to imC1q alone (CD163: p=0.030*) (Figure 14A-F, first/third vertical panel). No expression of CD80 and no differences in the expression levels of CD206 and CD273 could be detected independent of the priming conditions that were used (data not shown).

To mimic the inflammatory environment to which macrophages might be exposed in the tissue of SLE patients, an inflammatory signal was provided to the cells by adding LPS.

In response to LPS priming, HMDMs altered their phenotype by upregulating CD14, CD80, and CD274 (CD14: p=0.031*; CD80: p=0.0049**; CD274: p=0.013*). Furthermore, TLR4-triggering by LPS led to a downregulation of CD86 and CD163 (CD86: p=0.031*; CD163: p=0.040*). The expression of CD206 was unaffected by LPS (Figure 14A-F; second/fourth vertical panel).

HMDMs, which were incubated on imC1q in the presence of LPS, showed a trend to express higher levels of CD80 as compared to cells primed with LPS alone (CD80: p=0.063, ns). In contrast, combination of imC1q and LPS led to a decreased expression of CD163, CD274, and MHC class II (CD163: p=0.06, ns; CD274: p=0.05*; MHC class II: p=0.031*). Anti-C1q bound to imC1q in addition to TLR4-triggering by LPS increased the expression of CD80, CD274, and MHC class II as compared to imC1q and LPS (CD80: p=0.024*; CD274: p=0.016*; MHC class II: p=0.015*). Neither the expression of CD206 nor of CD273 was affected by bound anti-C1q as compared to imC1q in combination with LPS (Figure 14A-F; second/fourth vertical panel).

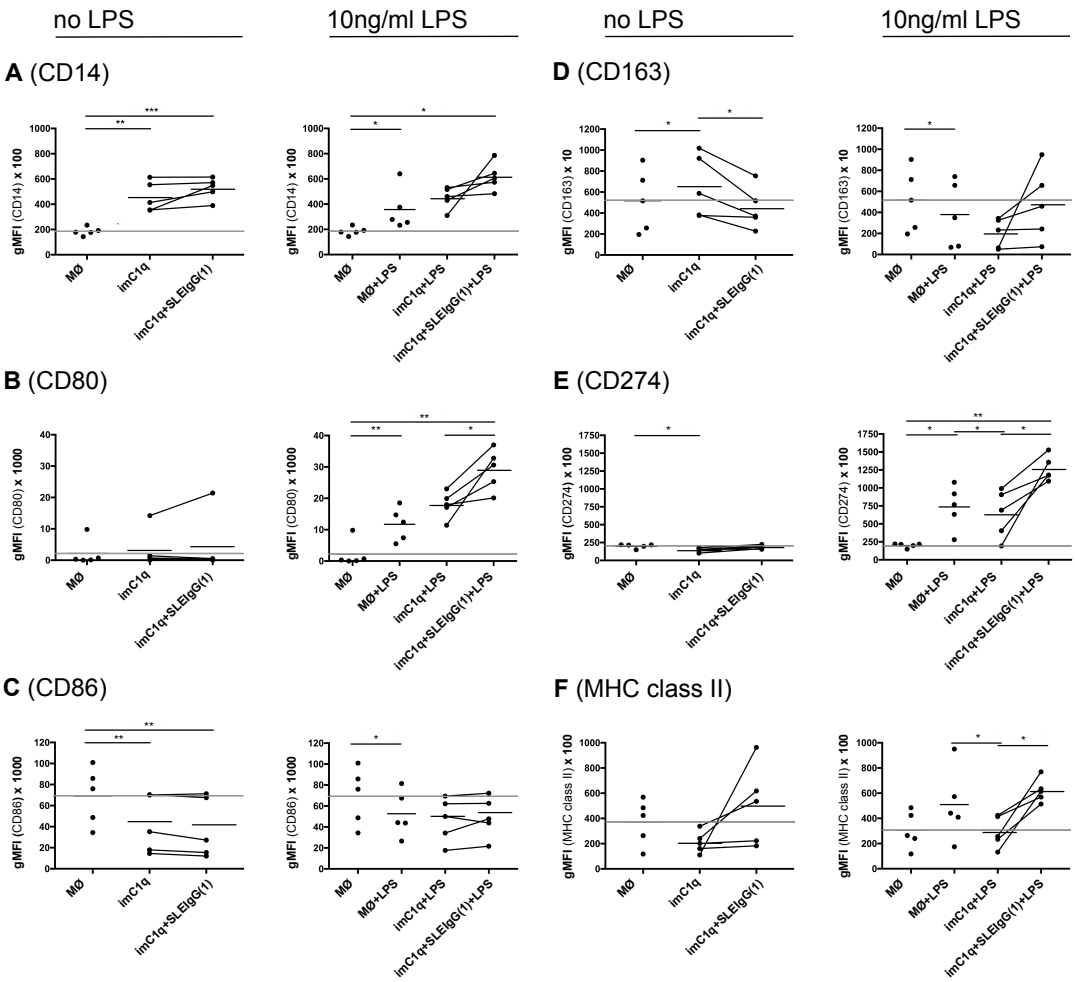


Figure 14: Phenotypic characterization of HMDMs primed on imC1q and bound anti-C1q.

Untreated HMDMs or HMDMs primed on imC1q alone or imC1q+SLEIgG(1) with or without 10ng/ml LPS for 18h were analyzed for their expression of surface markers by flow cytometry using conjugated antibodies (FITC-/PE-/APC-labeled antibodies) against CD14 (A), CD80 (B), CD86 (C), CD163 (D), CD274 (E), and MHC class II (F). The results of FACS analyses are expressed as gMFI (n=5). Grey lines represent mean gMFI values of untreated control cells. One-way ANOVA and Bonferroni's post-test, $p<0.05$*, $p<0.005$**, $p<0.0005$***.

Using anti-C1q from 2 additional SLE patients, i.e., SLEIgG(2) and SLEIgG(3), led to similar results (data not shown). In general, we could not observe a difference in the expression of surface markers by HMDMs primed on C1q-coated wells incubated with NHIgG as compared to those cells incubated on imC1q alone (data not shown).

Independent of the stimulation condition used, HMDMs exhibited macrophage characteristics such as the expression of CD14 and CD163, but no expression of DC-related markers, for example, no CD83 neo-expression upon LPS stimulation, could be detected.

In summary, HMDMs incubated on imC1q exhibited a $CD14^{high}$, $CD86^{low}$, $CD163^{high}$, $CD274^{low}$ phenotype being consistent with a M2-like phenotype. In the presence of LPS, imC1q priming led to a phenotype characterized by $CD14^{intermediate(int)}$, $CD80^{int}$, $CD274^{low}$, MHC class II^{low} expression comparable with traits of both M1 as well as M2. Furthermore, imC1q-bound anti-C1q priming resulted in a $CD14^{high}$, $CD86^{low}$, $CD163^{low}$, MHC class II^{high} phenotype consistent with a more M1-like phenotype. Combination of anti-C1q with TLR4-stimulation by LPS induced a $CD14^{high}$, $CD80^{high}$, $CD274^{high}$, MHC class II^{high} phenotype in HMDMs also consistent with a more M1-like phenotype.

Anti-C1q downregulate the endocytosis and phagocytosis rates of HMDMs

It is well known that C1q bound to apoptotic cells facilitates their uptake by phagocytes (23, 120). C1q might be concentrated in tissues due to local production by DCs and macrophages. Consequently, C1q might be present either in fluid-phase or deposited on cell surfaces in tissues (so-called tissue deposited C1q). Deposited C1q might have unique functions in serving as a priming agent for HMDMs or as a target for anti-C1q, thus influencing the ability of HMDMs to phagocyte dying cells (231).

Endocytosis is downregulated by anti-C1q

To investigate whether C1q and anti-C1q bound to imC1q affect the endocytic capacity of macrophages, primed HMDMs were incubated with conjugated FITC-dextran for 30 (Figure 15A) and 60min (Figure 15B). As expected, HMDMs primed with LPS alone slightly downregulated their ability to endocytose FITC-dextran as compared to untreated cells (30min: $p=0.061$, ns; 60min: $p<0.05$*). In contrast, imC1q priming alone slightly increased the endocytic activity of HMDMs as compared to untreated control cells (30min: $p=0.2305$, ns; 60min: $p=0.0793$, ns). Interestingly, HMDMs plated on bound anti-C1q derived from 2 different SLE patients reduced the endocytosis of FITC-dextran to the level observed in LPS-primed HMDMs (untreated vs imC1q+SLEIgG(1/2): 30min/60min: $p<0.05$*; imC1q vs imC1q+SLEIgG(1/2): 30min/60min: $p<0.001$**).

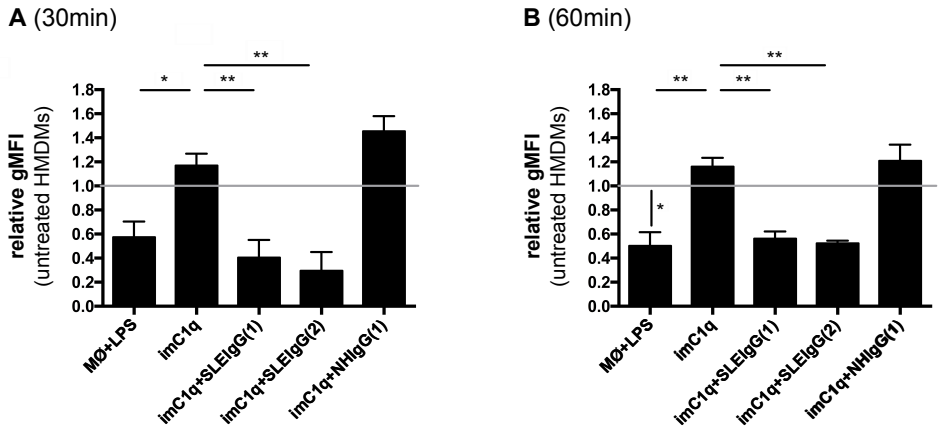


Figure 15: Endocytic activity of HMDMs is modulated by bound anti-C1q and imC1q.

After priming of HMDMs on imC1q, imC1q+SLEIgG(1/2), imC1q+NHIgG(1) or medium alone for 18h, cells were harvested, washed, and incubated with 0.5mg/ml FITC-dextran at 37°C (or 4°C) for 30 (A) or 60 (B) min. The uptake of FITC-dextran was analyzed by flow cytometry. The results are expressed as relative gMFI ± SEM of 3 independent experiments according to the following equation: relative gMFI=(gMFI(37°C)–gMFI(4°C))/gMFI(untreated HMDMs). Grey line shows mean gMFI values of untreated control cells. One-way ANOVA and Bonferroni's post-test, $p<0.05$*, $p<0.005$**.

Phagocytosis of apoptotic cells is differently modulated by imC1q and bound anti-C1q

Next, we investigated whether priming of HMDMs on imC1q as compared to cells being primed on bound anti-C1q has an effect on their phagocytosis rate. For this purpose, Jurkats were CFSE-labeled prior to UV-treatment to induce apoptosis. Early or late apoptotic Jurkats were then incubated with primed HMDMs (Figure 16). Early apoptotic cells were characterized by positivity for AnV and negativity for PI (Figure 16A; left side). Routinely, about 50-70% of early apoptotic cells were obtained. In contrast, late apoptotic cells were defined to be both AnV and PI positive (Figure 16A; right side).

In general, untreated HMDMs phagocytosed only low numbers of apoptotic Jurkats (early: 16.5%±4.3%; late: 13.4%±2.4%) (Figure 16B+C). In addition, we could not detect a significant difference when comparing the uptake of early to late apoptotic cells by any priming condition used. However, HMDMs primed with LPS downregulated their phagocytic ability as compared to untreated HMDMs (data not shown). Additional exposure to imC1q, on the other hand, led to an increased phagocytosis of both early and late apoptotic cells as compared to untreated control cells (early: 34.5%±7.3%, p=0.029*; late: 28%±1.3%, p=0.0057**). Furthermore, HMDMs incubated on anti-C1q bound to imC1q displayed a significantly lower phagocytosis rate of apoptotic cells as compared to imC1q-primed cells (early: 21.1%±4.3%, p=0.048*; late: 18.7%±2.2%, p=0.017*).

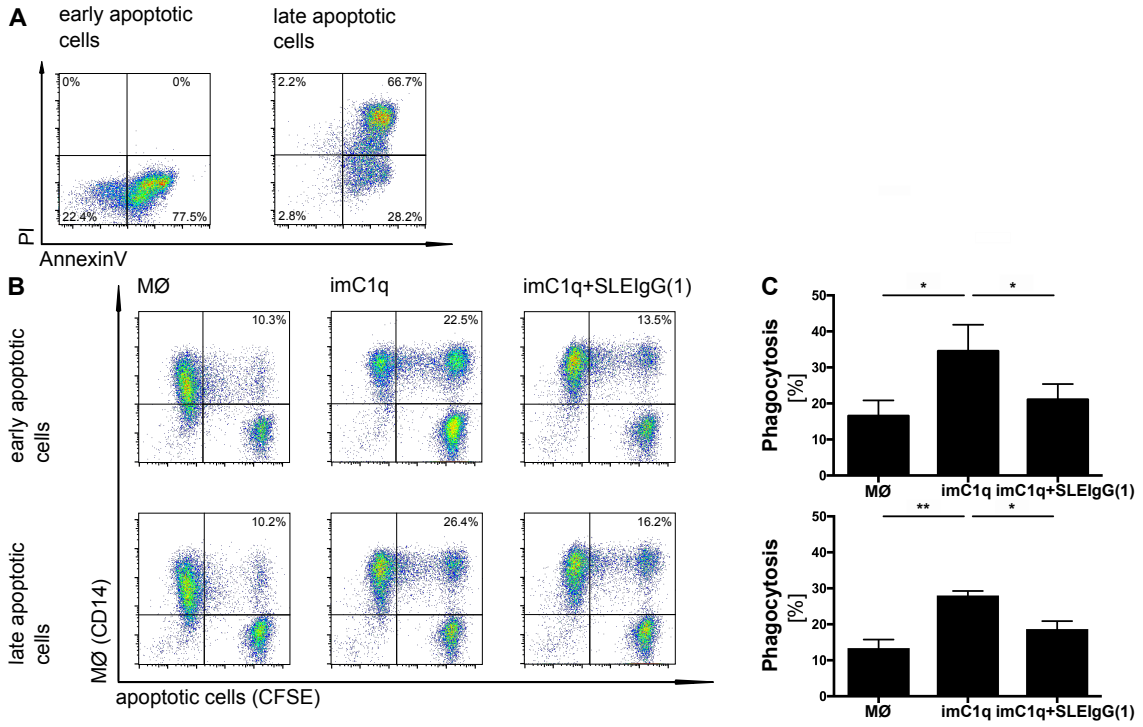


Figure 16: Phagocytosis rate of early and late apoptotic cells is downregulated by bound anti-C1q.

Apoptosis of Jurkats was induced by UV-treatment. Characterization of early and late apoptotic cells was performed by AnV- and PI-staining (A). CFSE-labeled early or late apoptotic cells were coincubated with differently stimulated HMDMs at a 1:1 ratio for 30min at 37°C. Unphagocytosed apoptotic cells were washed away and HMDMs were stained with an APC-conjugated mAB against CD14 (B). FACS dot-plots of one experiment being representative of 3 independent experiments are shown. Quantification of the uptake/adherence of apoptotic Jurkats was calculated as phagocytosis [%]=(($CFSE^+CD14^+$)/$CFSE^-CD14^+$)x100) (C). Results are shown as mean ± SEM of 3 independent experiments. One-way ANOVA and Bonferroni's post-test, $p<0.05$*, $p<0.005$**.

The downregulated phagocytosis rate of HMDMs incubated on imC1q-bound anti-C1q further underlines that anti-C1q might have a direct effect on HMDMs altering the clearance of apoptotic cells.

Anti-C1q downregulate MerTK expression

Because HMDMs primed on bound anti-C1q exhibited a lower phagocytosis rate as compared to cells stimulated on imC1q, we further analyzed MerTK expression by differently primed HMDMs (Figure 17). C1q can trigger an upregulation of MerTK in murine macrophages which was accompanied by an increased phagocytosis rate of apoptotic cells by macrophages (121). Additionally, it has been demonstrated that an efficient uptake of apoptotic cells by HMDMs is MerTK-dependent (232).

HMDMs incubated on imC1q upregulated MerTK as compared to untreated control cells (p=0.017*), whereas anti-C1q bound to imC1q suppressed MerTK expression as compared to imC1q (p=0.0048**) (Figure 17A). HMDMs stimulated with LPS also downregulated their MerTK expression as compared to control cells (p=0.012*) (Figure 17B). Again, in the presence of a proinflammatory stimulus imC1q slightly upregulated MerTK levels of HMDMs, whereas the combination of anti-C1q and TLR4-triggering by LPS slightly suppressed MerTK expression.

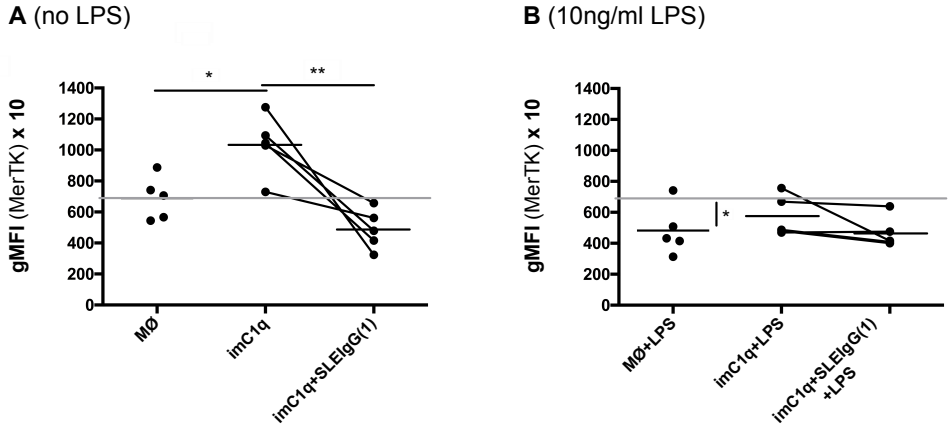


Figure 17: Anti-C1q suppress MerTK expression by HMDMs.

Untreated HMDMs or HMDMs primed on imC1q alone or on imC1q+SLEIgG(1) in the absence (A) or presence (B) of 10ng/ml LPS were analyzed for their expression of MerTK by flow cytometry. The results of FACS analyses are expressed as gMFI (n=5). Grey lines represent mean gMFI values of untreated control cells. One-way ANOVA and Bonferroni's post-test, p<0.05*, p<0.005**.

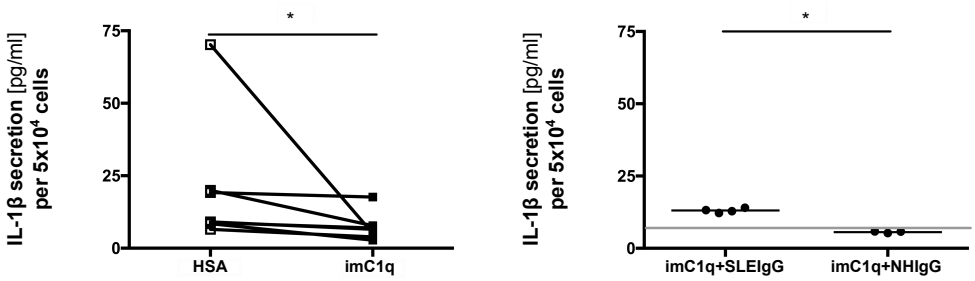
SLE patient-derived HMDMs exhibit a similar cytokine secretion profile as compared to HMDMs from healthy donors

So far, all experiments were performed using healthy donor-derived cells. We next studied cells derived from SLE patients at different stages of disease (Table 9), since cells of the monocyte-macrophage linage of SLE patients are known to exhibit functional defects (17, 18). The morphology of SLE patient-derived HMDMs incubated on different coatings followed a similar pattern as observed for healthy donor-derived HMDMs (data not shown).

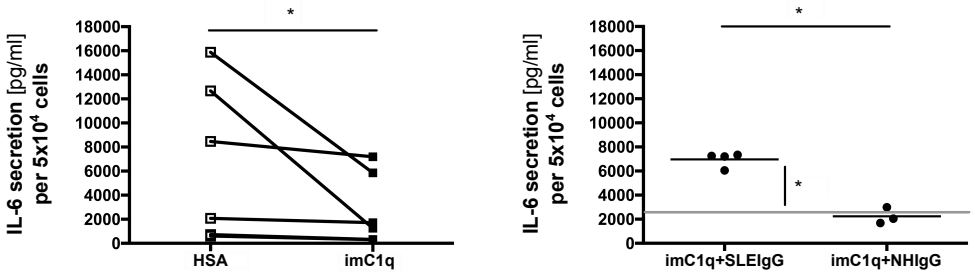
Similar to healthy donor-derived HMDMs, HMDMs obtained from SLE patients secreted lower LPS-induced levels of proinflammatory cytokines (IL-1β, IL-6, TNFα) when these cells were incubated on imC1q as compared to those stimulated on HSA (IL-1β: p=0.016*; IL-6/TNFα: p=0.031*) (Figure 18A-C; first horizontal panel). The anti-inflammatory effect of imC1q alone was abolished when SLE-derived HMDMs were incubated on anti-C1q bound to imC1q as evident by a significant upregulation of all proinflammatory cytokines tested as compared to imC1q+NHIgG (IL-1β/IL-6/TNFα: p=0.029*) (Figure 18A-C; second horizontal panel). Thus, these results were in accordance with the LPS-induced cytokines profiles of healthy donor-derived HMDMs (Figure 12A-C).

In addition, secretion of the anti-inflammatory cytokine IL-10 was also increased by cells being stimulated on imC1q as compared to those being incubated on HSA (IL-10: p=0.031*) (Figure 18D; first horizontal panel). However, when SLE patient-derived HMDMs were stimulated on anti-C1q bound to imC1q the cells significantly enhanced their LPS-induced IL-10 secretion as compared to imC1q+NHIgG (IL-10: p=0.029*) (Figure 18D; second horizontal panel).

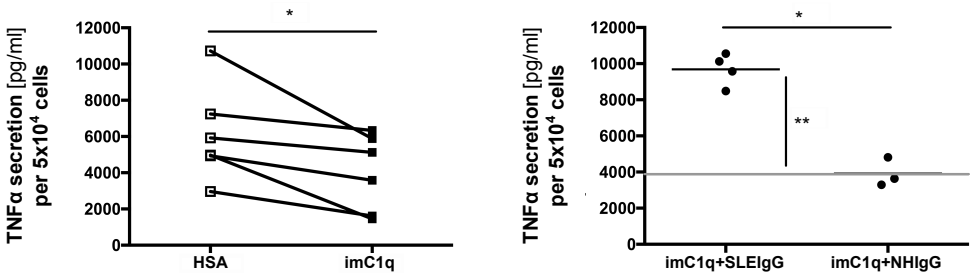
A (IL-1β)



B (IL-6)



C (TNFα)



D (IL-10)

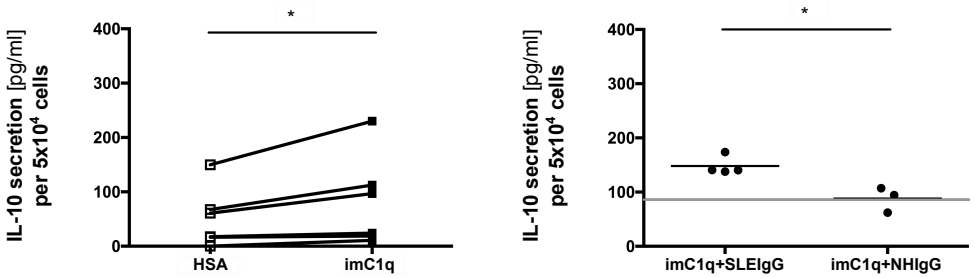


Figure 18: SLE patient-derived HMDMs show a similar cytokine secretion response as compared to HMDMs from healthy donors.

CD14-positive monocytes were isolated from SLE patients and differentiated into HMDMs. SLE HMDMs were incubated on different coatings for 18h and LPS-induced cytokine concentrations (IL-1β (A), IL-6 (B), TNFα (C), and IL-10 (D)) in SN were quantified. Data sets on the first horizontal panel show secreted LPS-induced cytokine levels of SLE HMDMs incubated on HSA (□) and imC1q (■). Data show cytokine release levels of 6 unrelated SLE patients. Wilcoxon-matched pair test, $p<0.05$*. Data sets on the second horizontal panel display LPS-induced cytokine levels secreted by SLE HMDMs adhered to imC1q+SLEIgG (n=4) or imC1q+NHIgG (n=2). Each data point represents pooled cytokine levels (mean) of 6 independent experiments. Grey lines represent mean cytokine levels secreted by SLE HMDMs incubated on imC1q alone. Mann-Whitney U test, $p<0.05$*, $p<0.005$**.

Interestingly, SLE macrophages also strongly reacted to the combination of self-anti-C1q bound to imC1q (data not shown). This phenomenon could be observed in the majority of patients and was dependent on anti-C1q levels measured in the serum of patients (Table 9).
Taken together, these results demonstrate that anti-C1q-triggered secretion of proinflammatory cytokines was similar between SLE- and healthy donor-derived HMDMs in the same experimental settings. However, LPS-induced IL-10 secretion was slightly increased in SLE-derived HMDMs when incubated on bound anti-C1q as compared to healthy donor control cells.

Discussion

Anti-C1q are believed to be pathogenic in SLE, in particular with regard to lupus nephritis. Anti-C1q have been found to deposit in glomeruli of patients with lupus nephritis and are believed to contribute to renal inflammation (7-10, 223, 225). Nevertheless, their biological and pathogenic properties in this inflammatory disease are not well defined. In this context, limited information was available about the effect of imC1q-bound anti-C1q on HMDMs. Here, we can now demonstrate that SLE patient-derived anti-C1q bound to imC1q induce a proinflammatory cytokine response as evident by an increased LPS-induced production of IL-1β, IL-6, and TNFα, as well as by a suppressed IL-10 secretion, thereby reversing the effect of imC1q alone. Additionally, bound autoantibodies induced a downregulation of CD163 and an upregulation of the LPS-induced expression of CD80, CD274, and MHC class II. In addition, HMDMs primed on anti-C1q bound to imC1q displayed a significantly lower phagocytosis rate of apoptotic cells accompanied by a reduced MerTK expression as compared to imC1q-primed HMDMs. Thus, bound anti-C1q altered the C1q-dependent suppression of macrophage-mediated inflammation by inducing a proinflammatory phenotype.

In analogy to the Th nomenclature, macrophage subsets have been classified as M1 and M2 subsets which are associated with different functions (184, 188, 189). However, translation of these macrophage phenotypes into disease models might be over simplified. *In vivo*, macrophages are constantly encountering various signals. Therefore, it might be possible that macrophages exhibit a phenotype showing both M1 and M2 characteristics and that multiple phenotypes coexist. Additionally, polarization of macrophages is thought to be partially reversible and in response to their microenvironment macrophages can express constantly changing phenotypes, also termed plasticity (184, 188). In fact, no characteristic macrophage phenotype could be defined in lupus nephritis. Different reports show that infiltrating macrophages/dendritic cells (DCs) in murine lupus nephritis are very heterogeneous (193, 194). Nevertheless, mononuclear cells play a role in the pathogenesis of organ related diseases, such as lupus nephritis, and are associated with chronic tissue damage and injury (195).

Infiltration of mononuclear cells plays a role in the progression of lupus nephritis and is associated with poor prognosis in SLE patients (233). Data from murine models of lupus nephritis suggest that macrophages and DCs infiltrating the kidneys display a heterogeneous group and are derived from circulating peripheral monocytes (193, 194). Sahu et al. found that the dominant macrophage subtype was neither M1 nor M2 and concluded that this phenotype might reflect an overall failure to resolve inflammation present in kidneys during flares (193). Additionally, the onset of proliferative glomerulonephritis in mice was associated with an

upregulation of chemokine and cytokine expression mediating further infiltration of activated DCs and monocytes into the kidneys. Macrophage subsets mainly secreted inflammatory cytokines (IL-1, IL-6, TNFα) and expressed CD11b, CD80, and CD86 confirming an activated macrophage phenotype. The authors suggested that the renal phenotype of macrophages resembles a M2b phenotype which are induced by FcγR ligation in addition to TLR-4 triggering by LPS (194). Others concluded that mononuclear phagocytes infiltrating the kidneys have an aberrant activation profile which contributes to the damage of renal tissue by mediating local inflammation as well as excessive tissue remodeling (234). We found that imC1q-bound anti-C1q induced the production of proinflammatory cytokines (IL-1β, IL-6, TNFα) accompanied by a significant upregulation of LPS-induced CD14, CD80, CD274, and MHC class II resembling more a M1-like phenotype.

Moreover, certain cytokines are implicated in the pathogenesis of SLE and lupus nephritis such as IL-6, IL-10, IL-17, type I interferons, and TNFα (235). In the context of a disease, cytokines are thought to play a role as mediators of inflammation and tissue damage. For example, in experimental IC glomerulonephritis, monocytes infiltrating the kidneys secret IL-1 which can trigger TNFα secretion and consequently leading to tissue injury (236). Additionally, urinary levels of IL-6 and IL-8 were higher in patients suffering from lupus nephritis as compared to patients without renal involvement or healthy controls suggesting a local production of these particular cytokines (237). Furthermore, analysis of isolated cell populations from nephritic mouse kidneys during flares demonstrated increased expression levels of IL-1, IL-6, IL-10, and TNFα in gene expression arrays (194). In addition to the anti-C1q-triggered increased production of IL-1β, IL-6, and TNFα, we found that LPS-triggered IL-10 secretion was suppressed in healthy donor-derived HMDMs when incubated on bound anti-C1q. In contrast, SLE patient-derived HMDMs showed an enhanced anti-C1q-triggered IL-10 production. Interestingly, serum levels of IL-10 are also elevated and even correlate with disease activity in SLE patients (203, 204). *In vitro*, both monocytes and B cells from SLE patients spontaneously secrete high IL-10 levels (238, 239). Based on these observations, IL-10 is considered to be involved in the pathogenesis of lupus (240). In the context of a chronically inflamed environment, it may be possible that IL-10's anti-inflammatory properties are lost and high IL-10 levels itself become pathogenic. Thus, SLE patient-derived HMDMs seem not only to have a defect in their phagocytosis efficiency but also in their cytokine response and thereby in the regulation of inflammation.

Beyond host defense, the complement system has an important function in the recognition and removal of apoptotic cell material. The efficient and fast clearance of dead cell material is crucial to avoid inflammatory and autoimmune processes (48, 226, 241). Moreover, C1q is an

essential molecule in the clearance of apoptotic cells by bridging apoptotic cells and phagocytes (22, 23, 120). In this context, it has been hypothesized that the binding of anti-C1q to C1q might interfere in the phagocytosis process. We can now demonstrate that bound anti-C1q suppress the C1q-dependent increased phagocytosis of apoptotic cell material by HMDMs. The downregulated phagocytosis rate of HMDMs incubated on imC1q-bound anti-C1q supports the hypothesis that anti-C1q have a direct effect on HMDMs by altering the clearance of apoptotic cells either by direct binding of C1q bound to apoptotic cells or by interfering indirectly with the uptake of apoptotic cells by binding to imC1q (e.g., deposited in tissues) and suppressing the clearance of apoptotic cells by inducing a less efficient phagocytic macrophage phenotype. It is well known that not all macrophage subsets display the same phagocytic efficiency. IL-10 producing macrophages, reflecting a M2-like phenotype, were found to preferentially clear early apoptotic cells and were more efficient in phagocytosis as compared to other subsets (242). We found that healthy donor-derived HMDMs being primed on imC1q were superior in clearing apoptotic cells and produced higher levels of IL-10 as compared to anti-C1q- and unprimed control cells.

C1q has a non-hepatic origin and is mainly produced by DCs and macrophages (26). In tissues, C1q might be accumulated during inflammatory processes due to local production by infiltrating DCs and macrophages (27, 28). The local synthesis and availability of this freshly synthesized C1q might exert different implications for local cells and their effector functions. It might even be possible that the release of C1q during the phagocytosis of apoptotic cells is upregulated which then in turn could increase, for example, MerTK expression itself and thus facilitate the clearance of apoptotic cells. Moreover, efficient uptake of apoptotic cells by HMDMs has been demonstrated to be MerTK-dependent (232). Additionally, 2 recent studies by Galvan et al. showed that imC1q can trigger an upregulation of MerTK expression and its ligand grow-arrest specific 6 in murine macrophages (121, 243). In this context, upregulation of MerTK expression was accompanied by an increased phagocytosis rate of apoptotic cells by macrophages (121). In analogy, we found that imC1q increased MerTK expression in HMDMs which correlated with an increased ability to phagocytose apoptotic cells. In contrast, bound-anti-C1q reduced MerTK expression which was accompanied by a lower phagocytosis rate. The reduced MerTK expression induced by anti-C1q might indicate an indirect mechanism by which these autoantibodies interfere in the uptake of apoptotic cell material by macrophages resulting in an increased apoptotic cell load. This indirect effect might even potentiate the direct effects on C1q. In fact, impaired and inefficient clearance of apoptotic material has been proposed as a mechanism underlying SLE pathogenesis causing an accumulation of dead cell material (20, 21).

An important limitation of our study is its *in vitro* character that not necessarily is reflecting the human *in vivo* situation. However, our experiments were performed using patient-derived high-affinity antibodies as described to correlate with disease activity (219) as well as patient-derived macrophages. In addition, our report on anti-C1q is in line with other studies demonstrating that autoantibodies from SLE patients can modify the response of phagocytes (154, 244, 245).

In conclusion, we can show that imC1q as well as bound anti-C1q skew the polarization of HMDMs into different phenotypes and that anti-C1q play a critical role as polarizing agent of HMDMs by inducing a proinflammatory phenotype and reversing the anti-inflammatory properties of imC1q alone. In addition, anti-C1q seem to directly and indirectly affect the phagocytic capacity of macrophages. Our results provide new insights into the pathogenic mechanisms of anti-C1q and their possible role in SLE.

Part 3

Anti-C1q autoantibodies from systemic lupus erythematosus patients induce C1q production by macrophages

Abstract

Antibodies against C1q (anti-C1q) are frequently found in patients with systemic lupus erythematosus (SLE). They strongly correlate with the occurrence of lupus nephritis and low circulating C1q levels. Previous studies demonstrated that myeloid cells, i.e., dendritic cells and macrophages, are a major source of C1q. However, a direct effect of anti-C1q on C1q secretion by macrophages has not yet been established. In the present study, we investigated the C1q secretion profile of human monocyte-derived macrophages (HMDMs) obtained from healthy donors and SLE patients *in vitro*. The effect of SLE patient-derived anti-C1q bound to immobilized C1q (imC1q) and imC1q alone on HMDMs was investigated by C1q secretion levels, the expression of membrane-bound and intracellular C1q using flow cytometry and ImageStream^X technology, and testing the ability of secreted C1q to activate the classical pathway (CP) of complement. Bound anti-C1q induced significantly higher C1q secretion levels as compared to imC1q alone or healthy donor IgG. The extent of C1q secretion by HMDMs correlated with IgG anti-C1q levels of SLE patients but not of healthy controls. Furthermore, bound autoantibodies and imC1q induced continuous and *de novo* C1q synthesis as evident by the intracellular C1q content, which correlated with C1q secretion levels. Finally, secreted C1q was able to activate the CP as reflected by C4b deposition. Interestingly, anti-C1q-dependent C1q secretion could also be observed in SLE patient-derived cells. In conclusion, our data indicate that imC1q-bound anti-C1q strongly stimulate the C1q production by HMDMs. Anti-C1q-induced C1q secretion might be an important immune-modulatory factor in SLE.

Introduction

Systemic lupus erythematosus (SLE) is characterized by B cell hyperactivity, production of a variety of autoantibodies directed against self-antigens including intra-cellular components and plasma proteins, formation of immune complexes (ICs), and ongoing complement activation and deposition resulting in inflammation and hypocomplementemia (44, 45).

Independently, homozygous deficiency of the complement protein C1q is the strongest known disease susceptibility gene for the development of SLE in humans, underlining that C1q plays a major role in the pathogenesis of SLE (3, 74). However, most SLE patients do not suffer from primary C1q deficiency, but aberrant complement activation is accounted for secondary hypocomplementemia. Low or undetectable C1q levels are frequently observed in SLE patients and are often associated with autoantibodies directed against C1q (anti-C1q). Anti-C1q are present in 20-50% of SLE patients and their occurrence correlates with both low complement levels as well as lupus nephritis (7-9). Even though, anti-C1q are clearly associated with active renal disease, the link between anti-C1q and low C1q antigen levels remains to be elucidated. In principle, anti-C1q could have an impact on complement activation as well as on C1q production.

C1q is mainly of a non-hepatic origin as opposed to most complement proteins which are produced as acute phase proteins in the liver (88). So far, different cell types have been described to produce and secret C1q, including epithelial cells and fibroblasts (96, 97). Nevertheless, it is believed that C1q is predominantly produced by myeloid cells (26, 68-70). This hypothesis is based on the fact that C1q-deficient mice, being devoid of any detectable serum C1q levels, were able to restore normal C1q serum levels upon receiving a bone marrow transplant from wild type mice, and vice versa (116). In addition, a patient suffering from homozygous C1q deficiency restored normal C1q serum levels after receiving a hematopoietic stem cell transplantation (246). Interestingly, monocytes fail to secrete C1q, even though, some cells have been tested positive for C1q mRNA (98). However, monocytes gain the ability to produce C1q during their differentiation into macrophages or dendritic cells (DCs) (26, 69). In particular, immature DCs (iDCs) are an important source of C1q, but upon maturation they downregulate this capability (26). In the circulation of healthy individuals C1q is mostly associated with its proteases C1s and C1r to form the C1 complex, the starter molecule of the classical pathway (CP) of complement (65, 66), whereas free C1q is mostly limited to the tissue. However, beyond complement activation, C1q has a dominant role in regulating inflammatory processes including autoimmunity. C1q has a major role in the clearance of apoptotic cell material (49). Different reports demonstrated that upon binding of C1q to apoptotic cells (76, 78), C1q facilitates the engulfment and clearance of apoptotic cells

(22, 23, 120), thereby limiting inflammation and autoimmunity during the phagocytosis of dead cell material. In this context, local synthesis of C1q in tissues by myeloid cells is believed to play an important role in the clearance of apoptotic cell material and in the recruitment of immune cells to sites of inflammation or injury in situ. Indeed, upregulated C1q production has been demonstrated in the brains of mice suffering from infections or Alzheimer's disease (130, 131), underlining the importance of local C1q production in the regulation of tissue homeostasis during inflammation.

We and others reported that immobilized (imC1q) induce an anti-inflammatory phenotype in macrophages (120, 247), whereas, on the other hand, anti-C1q bound to imC1q shifted the phenotype to a pro-inflammatory macrophage phenotype (247). So far, *in vitro* studies demonstrated that macrophages are also able to secrete C1q (26, 68-70). In addition, pro-inflammatory murine macrophages were demonstrated to produce more C1q as compared to their anti-inflammatory (resident) counterparts (27, 103, 248). Therefore, the aim of this study was to determine how SLE patient-derived high-affinity anti-C1q modulate the secretion of C1q by human monocyte-derived macrophages (HMDMs) and thus elucidate the association between anti-C1q and low serum C1q levels.

Patients and methods

Anti-C1q source/IgG source

A cohort of 30 SLE patients (Table 10) and 25 healthy control donors was investigated in this study.

Table 10: Characterization of SLE patients used as a source for anti-C1q.

Sex (females/males)	24/6
Age (years; median (range))	44 (29-77)
Hypocomplementemia (low C3 and/or C4) (yes/no) [1]	18/12
Lupus nephritis (yes/no) [1]	19/11
Positive for anti-C1q[1]	22
Negative for anti-C1q[1]	8

[1] Information at time point of blood sampling

All SLE patients fulfilled at least 4/11 criteria of the American College of Rheumatology (212, 213). Collection and use of serum samples were approved by the local Ethics Committee (EKZ-No.: 110/04; 130/05). IgG anti-C1q levels in SLE patients and healthy donors were measured and the O.D. values standardized and expressed as arbitrary units (AU) as described previously (249).

Isolation and differentiation of HMDMs

HMDMs were isolated and differentiated as described previously (S. Thanei et al. (submitted)). Briefly, monocytes were purified by positive selection with CD14 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from peripheral blood mononuclear cells isolated by Ficoll-Paque (Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA)) density gradient centrifugation (consistent purities of >95% of CD14-positive monocytes). CD14-positive monocytes were then cultured in Dulbecco modified essential medium supplemented with 1% penicillin/streptomycin (DMEM+; both from Life Technologies, Invitrogen, Carlsbad, CA, USA) and 10% normal human sera (pooled from 40 healthy donors). The culture was maintained in 5% CO_2 at 37°C for 7 days and the media were exchanged every 2-3 days.

Macrophages obtained from SLE patients

15ml of heparinized venous blood was obtained from SLE patients (Table 11) (EKZ-No.: 2014/125).

Table 11: Characterization of SLE patients from whom CD14-positive monocytes were isolated[1].

Pat ID (sex (f/m))	**Age** (y)	**Disease duration** (y)	**Anti-C1q levels** (cut-off: 98AU)	**Disease state**	**Medication**
SLE(A) (f)	66	0	342AU	flare (stroke; LN)	Pred
SLE(B) (f)	31	12	203AU	R (LN (class IV))	AZA, Pred, RIT
SLE(C) (m)	30	7	92AU	R	HCQ
SLE(D) (f)	48	20	51AU	R (LN (class IV))	MMF, Pred
SLE(E) (f)	34	0.5	1'080AU	R (no LN)	MMF
SLE(F) (m)	40	14	73AU	R	Pred

Legend: AU: arbitrary units; AZA: Azathioprine; f: female; HCQ: Hydroxychloroquine; LN: lupus nephritis; m: male; MMF: Mycophenolate mofetile; pat: patient; Pred: Prednisone; R: remission; RIT: Rituximab; y: years;

[1] Information at time point of blood sampling

All these patients fulfilled at least 4/11 criteria of the American College of Rheumatology (212, 213). CD14-positive monocytes were isolated and differentiated as described above with one exception. Adherent monocytes were differentiated into macrophages in DMEM+ supplemented with 10% autologous serum for 7days. For the analysis of secreted C1q levels, the patient's differentiated macrophages were stimulated as described below. For the stimulation, the patient's own anti-C1q were included in the experimental setting too.

In vitro stimulation model for HMDMs

After 7 days, HMDMs were harvested using ice-cold PBS (Life Technologies), resuspended in DMEM+ supplemented with 0.1% human serum albumin (HSA; HSA-DMEM; Sigma-Aldrich) at $0.5x10^6$cells/ml, and used for stimulation experiments. Therefore, 96well plates (MaxiSorp, NalgeNunc International, Rosklide, Denmark) were coated with C1q (immobilized C1q (imC1q); Complement Technology, Tyler, TX, USA) or HSA at 5µg/ml in coating buffer (0.1M sodium carbonate buffer, pH 9.6) overnight at 4°C. Plates were washed twice with PBS and C1q-coated wells were incubated with 100µg/ml purified total IgG (purified by ProteinG affinity columns) from SLE patients or healthy donors diluted in high-salt buffer (PBS containing 1M NaCl) for 1h at 37°C. Before use, each IgG preparation was centrifuged for 30min at 14'000xg at 4°C. After washing plates 4times with PBS, HMDMs (100µl/well of $0.5x10^6$cells/ml suspension) were added to the wells, cells were allowed to adhere for 60min at 37°C, and 10ng/ml lipopolysaccharide (LPS; *Escherichia coli*: O127:B8; Sigma-Aldrich) or DMEM-HSA only were added.

For reseeding experiments, cells were stimulated as described above, harvested after 22h, washed intensively with PBS, and reseeded into 96-well plates at $0.5x10^6$cells/ml (50'000cells/200µl). Supernatants (SN) were collected as indicated.

Quantification of secreted C1q in SN by ELISA

After stimulation of HMDMs on different coatings for 22h, SN were collected, centrifuged, and kept at -80°C until analysis. The concentrations of C1q were measured using a sandwich ELISA as described below. All samples were analyzed in duplicates.

Description of the ELISA

A specific sandwich ELISA was developed for the detection of secreted C1q in SN. As capturing antibody a mouse monoclonal C1q-specific antibody (mAB: clone 34A4: (153)) was coated over night at 4°C in coating buffer. Plates were blocked using PBS containing 3% BSA (assay diluent (AD)) for 1h at room temperature (RT). To generate a standard curve, purified C1q was used in a range of 0-25ng/ml diluted in AD. After adding samples and standards, and incubating for 2h at RT, a goat anti-human C1q (Quidel, San Diego, CA, USA) was used followed by a HRP-labeled anti-goat IgG (Sigma-Aldrich). Both antibodies were diluted in AD and were incubated for 1h at RT. After every incubation step, plates were washed 3times with washing buffer (PBS-T; PBS containing 0.05% Tween). Enzyme activity was assessed by addition of TMB substrate (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Absorbance was read at 450nm using a microplate-biokinetics reader (BioTek Instruments, Winooski, VT, USA). The detection limit of the ELISA was 0.01ng/ml.

The specificity of the ELISA for C1q was confirmed by measuring C1q in sera of 5 different healthy donors (range: 42-118µg/ml C1q). Additionally, C1q-depleted sera (C1qDS) and C1q-deficient sera emitted no signal. To exclude unspecific C1q binding to coated mouse IgG, we used 2 control mABs (namely clone 24C4 and 36A10; showing no binding to C1q) as capture antibodies. No detectable signal could be measured when these coated mABs were incubated with different concentrations of purified C1q and compared to the C1q-specific mAB (clone 34A4), generally used as capture AB. Levels of C1q were undetectable in identical C1q-coated control wells that did not contain phagocytic cells, suggesting that the detected C1q was not the result of contaminating coating protein. Furthermore, to exclude degradation of imC1q by HMDMs, biotinylated C1q (biot-C1q) was coated and HMDMs were stimulated as described above. SN were then tested for biot-C1q using the sandwich ELISA described above with a minor modification. The ELISA was developed for captured biot-C1q with Streptavidin conjugated-HRP. No signal was obtained from this construct, suggesting that C1q was not detached from plates. Also, CD14-positive monocytes incubated on imC1q did not lead to detectable levels of secreted C1q (data not shown).

Detection of membrane-bound C1q and intracellular C1q by flow cytometry and ImageStreamX flow cytometry

Flow cytometry

After stimulation of HMDMs on different coatings for 22h using the *in vitro* model described above, cells were collected and washed twice with FACS buffer (PBS containing 1% BSA and 1mM sodium azide). Finally, cells were resuspended in FACS buffer at $1x10^6$cells/100µl. Non-specific binding to Fcgamma receptors (FcγRs) was blocked by incubating cells with 2mg/ml human IgG in 100µl FACS buffer/$1x10^6$ cells for 45min at 4°C. For FACS analysis of membrane-bound C1q (mC1q), cells were resuspended at $5x10^5$cells/100µl in FACS buffer and stained using a rabbit anti-C1q conjugated with FITC (Dako, Baar, Switzerland) or its isotype-control, a rabbit IgG conjugated with FITC (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), for 45min at 4°C. For intracellular C1q (iC1q) staining, cells were first fixed using PBS containing 4% paraformaldehyde for 20min on ice, washed, and permeabilized with FACS buffer containing 0.01% saponin (permeabilization buffer) on ice for 10min. Cells were then incubated with either a FITC-labeled anti-C1q or its matched isotype control for 45min at 4°C. Both antibodies were diluted in permeabilization buffer. After washing cells twice, 10'000 events were acquired for each dataset using a FACSAccuri (BD Biosciences) and analyzed using Flowjow software (Tree Star Inc., Ashland, OR, USA). The final geometric mean fluorescence intensities (gMFI) were calculated by subtracting the gMFI of the corresponding isotype control from the gMFI of the sample.

ImageStream^X flow cytometry

For iC1q detection, primed HMDMs were stained as described above. Stained cells were analyzed using an ImageStream^X flow cytometer and IDEAS image analysis software package (both developed by Amnis, Seattle, WA, USA). 5'000 single cell images per sample were collected and single-color compensation controls (each 500 cells) were used to generate a compensation matrix that was applied to the image data to correct for spectral overlap as suggested by the manufacturer. The data file of each stimulation condition was then processed through a single template using a consistent gating strategy first gating on single cells (by area aspect ratio) and then on focused cells (by gradient root mean square of the brightfield image). The stored content of iC1q was quantified using the spot count feature. Data are expressed according to the following method: iC1q expression [%] (=number of spots/cell) were defined as low (0-15 spots/cell), as intermediate (15-30 spots/cell), or as high C1q expression (>30 spots/cell), respectively. There was no significant colocalization between

the intensity of iC1q with neither the cell nucleus (DAPI) nor the cell membrane (Dao far red) within HMDMs independent of the priming condition used (data not shown).

Functional analysis of C1q produced by HMDMs

Procedure for C1q-containing SN

SN from HMDMs (stimulation experiments) were collected and pooled from 6 different donors in order to obtain sufficient amounts of C1q for functional tests. These SN were further processed as following: First, SN were concentrated by ammonium sulfate precipitation at a saturation of 40% on ice with stirring for 4h. The precipitate was collected by centrifugation at 25'000xg for 60min at 4°C. The pellet was dissolved in dialysis buffer (10mM Hepes and 0.3M NaCl, pH 7.2) and dialyzed 3times against the same buffer overnight at 4°C (modified according to (100)).

Activation of the CP using an IgM-coated plate

Functional activity of secreted C1q in the activation of the CP was analyzed by ELISA using IgM as the ligand. ELISA plates were coated with human IgM (2µg/ml; Sigma-Aldrich) in coating buffer overnight at 4°C. After each incubation step, plates were washed 3times with PBS-T. Residual binding sites were blocked with BSA-PBS for 1 hour at RT. Then, the plates were incubated with C1qDS only or C1qDS reconstituted with either purified C1q (concentration corresponding to the concentration measured in purified SN) or with C1q obtained from concentrated SN. All samples were serially diluted 1:1-1:8 in 10% C1qDS diluted in veronal buffered saline (VBS: 5mM barbituric acid, 0.5mM $MgCl_2$, 2mM $CaCl_2$, 140mM NaCl, and 0.05% Tween, pH 7.5) and incubated for 1 hour at 37°C. After washing, deposited C4b was detected by a goat-anti C4b (Complement Technology). As a secondary antibody a HRP-labeled anti-goat IgG was used. After a final washing step, the enzyme activity of HRP was detected as described above.

Statistical analysis

Data are expressed as mean ± SEM if not stated otherwise. Statistical analyses were performed using Mann-Whitney U test to compare 2 groups. To compare more than 2 groups, one-way or two-way ANOVA followed by Bonferroni's post-test were used as indicated. Correlations were calculated using Spearman's rank correlation coefficient (R). Data were analyzed using GraphPad Prism software (GraphPad Prism, CA, USA). A p value <0.05 was considered statistically significant.

Results

Anti-C1q bound to imC1q induce C1q secretion by HMDMs

Most complement proteins have a hepatic origin, except C1q which is predominantly produced by myeloid cells (26, 68-70). *In vitro*, its production can be modulated by different agents, including LPS, steroids, cytokines, and ICs (27, 104, 119).

In order to study if anti-C1q bound to imC1q influence C1q secretion by HMDMs, we first incubated HMDMs on different coatings, including HSA, ICs (consisting of human anti-BSA IgG-BSA), purified total IgG from a healthy donor (NHIgG(1)) and from an anti-C1q positive SLE patient (SLEIgG(1)), as well as mannose-binding lectin (MBL), C1 complex, denatured C1q (denat. C1q; C1q stock-solution denatured at 56°C for 30min), and imC1q (Figure 19A). Most of these coated proteins did not induce C1q secretion or only to a low degree. However, imC1q itself increased C1q secretion by HMDMs (for example, imC1q vs HSA: $p<0.05$*). To induce a mild pro-inflammatory environment to which macrophages might be exposed in the tissue of SLE patients, an additional inflammatory stimulus was provided to HMDMs by adding LPS. This led to an increased C1q secretion as compared to imC1q-coating alone (imC1q vs imC1q+LPS: $p<0.05$*).

Next, we assessed whether anti-C1q bound to imC1q (imC1q+SLEIgG) influence C1q secretion levels of HMDMs as compared to imC1q alone (Figure 19B). Interestingly, imC1q+SLEIgG upregulated C1q secretion levels as compared to imC1q alone or C1q-coated wells incubated with control IgG (imC1q+NHIgG) (imC1q+SLEIgG vs imC1q: $p<0.005$**; vs imC1q+NHIgG: $p<0.001$***).

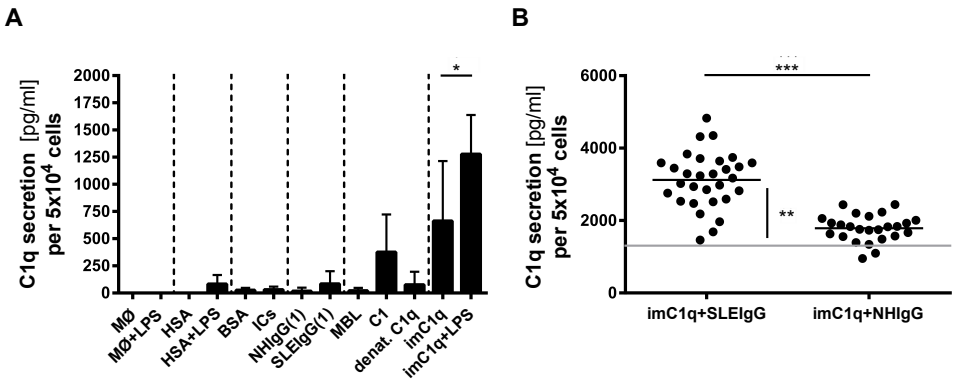


Figure 19: Modulation of C1q secretion by HMDMs incubated on imC1q and anti-C1q bound to imC1q.

HMDMs were incubated on different coatings for 22h: left untreated (MØ) or incubated on HSA, ICs (BSA–human anti-BSA IgG) or BSA only, coated purified total IgG (50µg/ml) obtained from a healthy donor (NHIgG(1)) or from an anti-C1q positive SLE patient (SLEIgG(1)), or MBL, C1 complex, denat. C1q, or on imC1q alone with or without LPS (all proteins were coated at 5µg/ml if not stated otherwise) (A). Data show the mean ± SEM of 5 different donors. One-way ANOVA including Bonferroni's post-test, $p<0.05$*. HMDMs were incubated on imC1+SLEIgG (n=30 different IgG preparations) or as a control on imC1q+NHIgG (n=25 different IgG preparations) for 22h (B). Each data point represents pooled C1q secretion levels (mean) of 5 independent experiments. Grey line represents mean C1q secretion levels secreted by HMDMs incubated on imC1q alone. Mann-Whitney U test, $p<0.005$**, $p<0.001$***.

Furthermore, the C1q levels secreted by HMDMs correlated with IgG anti-C1q levels of SLE patients (n=30; R=0.5595; p=0.0013**) (Figure 20A). No such correlation was observed in healthy donors (n=25; R=0.3123; p=0.1285; ns) (Figure 20B).

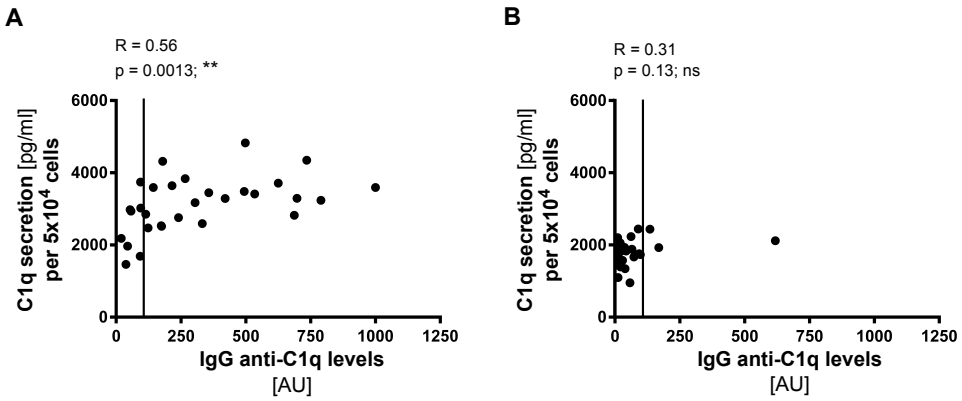


Figure 20: Correlation of IgG anti-C1q levels with C1q secretion by HMDMs in SLE patients and healthy donors.

IgG anti-C1q levels of SLE patients and healthy donors were plotted against C1q secretion levels of HMDMs obtained from healthy donors. The extent of C1q secretion induced in HMDMs correlated with IgG anti-C1q levels of SLE patients (A) but not of healthy donors (B). Anti-C1q levels are expressed relative to a standard serum as described in patients and methods. Vertical lines indicate the cut-off values for positive IgG anti-C1q levels. Spearman's rank correlation coefficient (R); $p<0.005$**.

In contrast, 2 anti-C1q control mABs (clones 34A4 and 12F10 (153)) bound to imC1q did not induce a significant upregulation of C1q secretion as compared to imC1q alone (data not shown), and a polyclonal goat anti-C1q bound to imC1q completely abolished C1q secretion (data not shown). However, it needs to be considered that both antibodies were not of human origin (as indicated).

In contrast to imC1q alone, the addition of LPS to HMDMs incubated on imC1q-bound anti-C1q derived from different SLE patients did not significantly alter C1q secretion (data not shown).

To exclude the possibility that a potential LPS contamination in the HSA, C1q, or SLEIgG preparations could be responsible for the stimulation of HMDMs inducing the secretion of C1q, we used Polymixin B to block LPS. However, the C1q secretion profile was unaffected by the presence of Polymixin B indicating that a LPS contamination is unlikely (data not shown).

Even though, we obtained consistent C1q secretion profiles, secreted C1q levels varied between HMDMs derived from different healthy donors which seems to be in line with the fact that C1q serum levels also vary in healthy individuals (range: 70-250µg/ml (67)).

Taken together, these data indicate that SLE patient-derived bound anti-C1q can induce C1q secretion by HMDMs.

Anti-C1q induce continuous and cumulative synthesis of C1q by HMDMs

C1q has been shown to be continuously and cumulatively secreted by macrophages when kept in cultures for several days (69). To evaluate the kinetics of C1q secretion by differently stimulated HMDMs, cells were incubated on HSA, imC1q, imC1q+SLEIgG(1), or imC1q+NHIgG(1). SN were collected every 24h and analyzed for secreted C1q levels.
Analyzing C1q secretion over 6 days, we observed that C1q is continuously secreted by HMDMs (Figure 21/ Table 12).

Table 12: Summary of the kinetics of C1q secretion by HMDMs.

Stimulation conditions	**C1q secretion** [pg/ml] **during stimulation time** [days]		
	1day	**2days**	**6days**
HSA	132.7±229.8	198.2±245.6	1'019±643.5
imC1q	1'203±677.5	2'088±878.5	5'332±2'727
imC1q+SLEIgG(1)	2'230±1'052	3'822±1'617	7'295±2'651
imC1q+NHIgG(1)	1'586±892.7	2'643±500.7	6'003±1'072

Shown are mean values ± SEM of 3 independent donors.

In addition, we detected a linear kinetic of C1q secretion by HMDMs independent of the stimulation condition used. HMDMs adherent to HSA continuously secreted low levels of C1q, whereas, in general, HMDMs incubated on imC1q produced continuously higher C1q levels. We did not observe a significant difference in the kinetics of C1q secretion by HMDMs incubated on imC1q as compared to imC1q+NHIgG(1). However, when HMDMs were incubated on imC1q+SLEIgG(1), the cells secreted significantly higher C1q levels within the first 24h of culture and throughout the whole incubation time of 6 days as compared to all other stimulation conditions used. Interestingly, cells incubated on both, imC1q as well as bound-anti-C1q, reached a plateau in their C1q production on day 5. This plateau of C1q secretion was not due to increased cell death since double-staining for AnnexinV and Propidium iodide did not show any significant difference between the different priming conditions (data not shown).

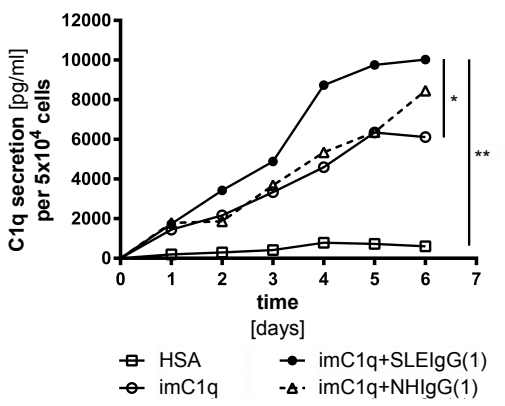


Figure 21: Kinetics of C1q secretion by HMDMs incubated on different coatings.

HMDMs were incubated on HSA, imC1q, or imC1q+SLEIgG/NHIgG for up to 6days. SN were collected at different time points as indicated in the figure and secreted C1q levels were measured by ELISA. Shown are the results of one donor used to obtain HMDMs being representative of 3 independent experiments. Two-way ANOVA including Bonferroni's post-test (vs imC1q+SLEIgG(1)), $p<0.05$*, $p<0.01$**.

Thus, independent of the stimulation condition used, HMDMs continuously secreted C1q. These data suggest that bound anti-C1q can induce continuously high C1q production levels in macrophage-riche tissues where anti-C1q are deposited.

Prolonged effect of C1q secretion by HMDMs stimulated on imC1q and anti-C1q bound to imC1q

Next, we assessed whether the increased C1q secretion by imC1q and imC1q-bound anti-C1q is sustained in HMDMs after withdrawal of the initial stimulus. After stimulation on different coatings, SN were collected and cells were washed and reseeded into fresh uncoated culture plates (Figure 22).

In accordance with previously described data (Figure 19), untreated HMDMs secreted consistently low C1q levels, whereas cells being incubated on imC1q+SLEIgG(1) secreted significantly more C1q as compared to imC1q-primed HMDMs (data not shown).

24h after reseeding, C1q secretion levels decreased to low or undetectable levels dependent on the coating condition used before (Figure 22). However, 3days after reseeding, cells being initially primed on imC1q+SLEIgG(1) secreted significantly higher levels of C1q as compared to the other priming conditions (imC1q+SLEIgG vs untreated: $p<0.01$**; vs imC1q: $p<0.05$*). Strikingly, 5days after reseeding, HMDMs primed on imC1q secreted significantly higher C1q levels as compared to untreated control cells ($p<0.001$***). Nevertheless, the highest C1q levels were produced by HMDMs primed on bound anti-C1q before being reseeded and kept in culture for 5days (imC1q+SLEIgG vs untreated: $p<0.001$***; vs imC1q: $p<0.01$**).

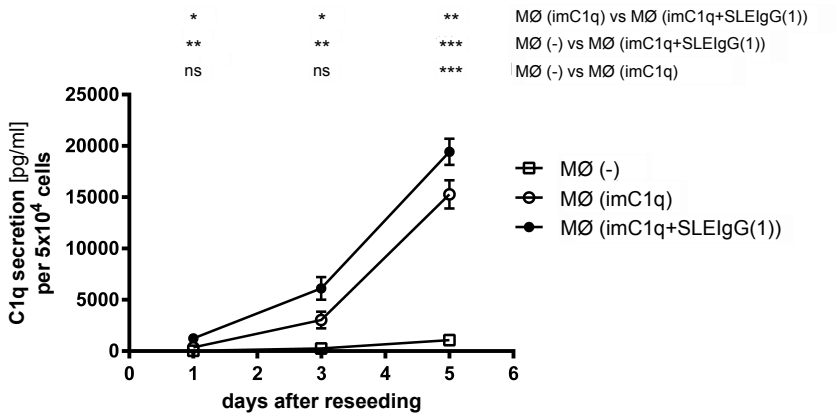


Figure 22: Prolonged effect of C1q secretion in stimulated HMDMs.

HMDMs were incubated either on HSA, imC1q, imC1q+SLEIgG, or imC1q+NHIgG. After 22h, SN and cells were collected. Cells were washed, resuspended in fresh medium, reseeded, and further incubated for indicated time points. Results are shown of C1q secretion levels by HMDMs after being reseeded into new culture dish (without the initial coating) for 1, 3, or 5 days. Data represent pooled C1q secretion levels (mean) ± SEM of 5 unrelated healthy donors used to obtain HMDMs. One-way ANOVA including Bonferroni's post-test, $p<0.05$*, $p<0.01$**, $p<0.001$***.

Anti-C1q bound to imC1q induce high iC1q storage and *de novo* C1q synthesis

Since we observed that both imC1q as well as imC1q-bound anti-C1q induced an increased C1q secretion by HMDMs, we sought to determine if differently primed HMDMs express different levels of membrane-bound C1q (mC1q) or intracellular C1q (iC1q). Furthermore, we analyzed the stored content of iC1q by ImageStream^X flow cytometry.

mC1q has been described as a feature of macrophages and earlier reports demonstrated that both HMDMs and DCs exhibit low expression of mC1q (250, 251). In contrast, Bensa et al. could not detect mC1q on unstimulated macrophages (69).

Independent of the stimulation conditions used, we observed only minor expression of mC1q on the surface of HMDMs derived from 3 out of 5 different healthy donors (Figure 23A). The low expression levels of mC1q were not due to the detecting antibody that was verified to be able to detect surface-bound C1q (data not shown). In addition, expression of mC1q did not increase during extended observations as outlined in the paragraph before.

To evaluate whether differently primed HMDMs express different iC1q levels, we used flow cytometry and ImageStream^X flow cytometry technologies.

HMDMs incubated on imC1q significantly upregulated their iC1q expression as compared to untreated control cells which expressed low levels of iC1q ($p<0.05$*) (Figure 23B). In addition, we observed a slightly increased iC1q expression by HMDMs being incubated on bound anti-

C1q derived from one SLE patient when compared to cells stimulated on imC1q alone (p=0.063; ns). HMDMs stimulated with the TLR4-ligand LPS displayed higher expression of iC1q as compared to untreated cells (data not shown). The findings of the stored iC1q content are consistent with C1q levels being secreted by HMDMs from the same donor (Figure 23C).

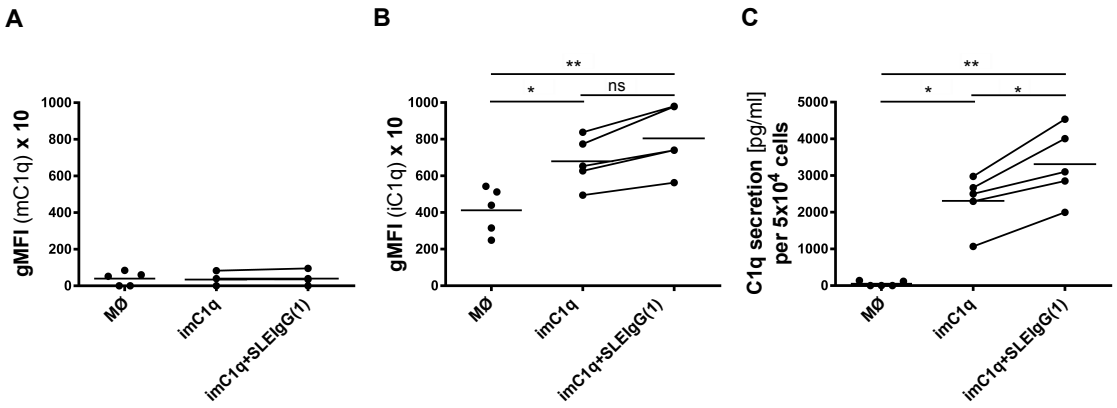


Figure 23: Expression of mC1q and iC1q by differently primed HMDMs detected by flow cytometry.

HMDMs were left untreated or stimulated on imC1q alone or imC1q+SLEIgG(1). After 22h of stimulation on different coatings, SN and cells were collected. Cells were washed and stained for mC1q (A) or iC1q (B) as described in patients and methods. The results of FACS analyses are expressed as gMFI (n=5). C1q secretion levels in SN (C) from the same HMDM donors were analyzed in parallel. Results are shown as mean ± SEM of 5 independent experiments. Lines between data points indicate that the cells were derived from the same donor. One-way ANOVA including Bonferroni's post-test, $p<0.05$*, $p<0.005$**.

Using anti-C1q from 2 additional SLE patients, i.e., SLEIgG(2) and SLEIgG(3), led to similar iC1q expression levels (data not shown). Furthermore, we could not observe a difference in iC1q expression by HMDMs primed on C1q-coated wells incubated with NHIgG as compared to those cells incubated on imC1q alone (data not shown).

Using ImageStream^X flow cytometry, we next evaluated whether C1q is freshly synthesized or released from intracellular storage pools.

Untreated HMDMs had only a small amount of iC1q stored intracellularly. Moreover, not all cells stained positive for iC1q (Figure 24A; first 2 horizontal panels; Figure 24B; upper histogram) confirming findings reported previously (99). Nevertheless, we observed brighter storage pools in HMDMs being incubated on imC1q and even more in cells stimulated on imC1q+SLEIgG(1) (Figure 24A; third-sixth horizontal panels; Figure 24; middle/lower histograms). When quantifying iC1q in HMDMs primed on different coatings (Figure 24C), untreated HMDMs had mostly low expression levels of iC1q and only a low percentage of

cells had intermediate or high iC1q expression levels. HMDMs primed on imC1q had mostly intermediate iC1q expression levels, whereas HMDMs primed on bound anti-C1q displayed mostly high iC1q expression levels.

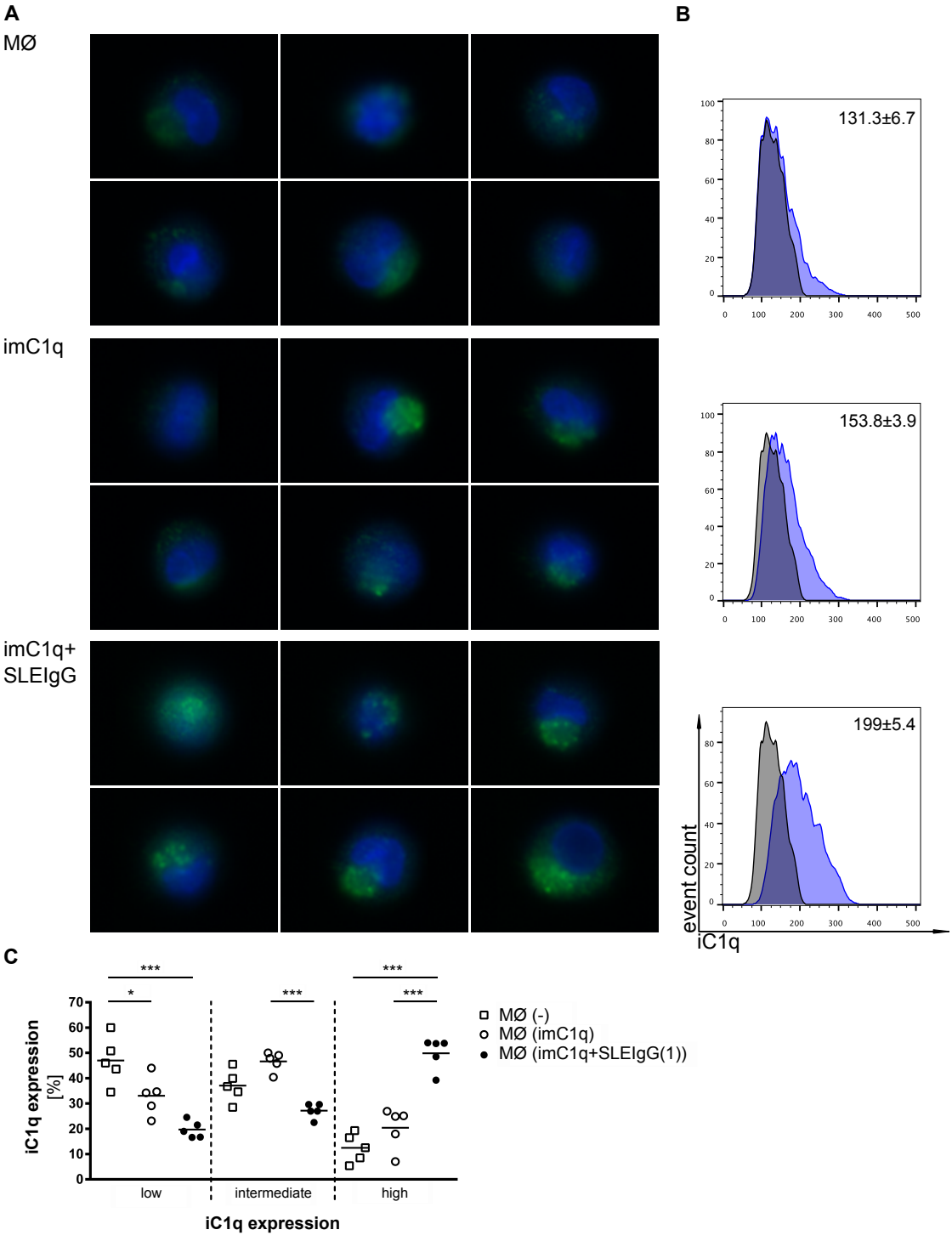


Figure 24: Intracellular content of C1q correlates with C1q secretion levels.

HMDMs were stimulated and stained as described in patients and methods. Stained cells were analyzed using an Amnis ImageStream^X instrument and cell images were processed using IDEAS software. Representative images of differently stimulated HMDMs captured by the Amnis ImageStream^X system and stained with C1q (green) and DAPI (blue) are shown (A). Shown are 6 single cell images per priming condition from one experiment being representative for 5 independent experiments. Histograms show iC1q intensities (blue) and isotype controls (grey) for each priming condition (B). Mean gMFI values ± SEM are indicated for each priming condition in the corresponding histogram. Shown is one histogram per priming condition from one experiment being representative for 5 independent experiments. Different levels of iC1q were calculated using a template provided by the IDEAS software package and as described in patients and methods (C). One-way ANOVA including Bonferroni's post-test, $p<0.05$*, $p<0.01$**, $p<0.001$***.

These data indicate that high C1q secretion levels are associated with an accumulation of iC1q, indicating that anti-C1q and imC1q upregulate both, the secretion as well as the secretion of C1q.

C1q secreted by HMDMs is functional

C1q produced by macrophages and iDCs is able to activate the CP of complement (26, 70). In order to verify that secreted C1q is functional, we analyzed its complement-activating potential in a CP-activation assay using IgM-coated ELISA plates.

Serial dilutions of concentrated culture SN of stimulated HMDMs diluted in C1qDS/VBS and incubated on IgM-coated plates resulted in the activation of the CP as assessed by C4b deposition (Figure 25), indicating that HMDMs secret functional and intact C1q. In accordance with the observed C1q secretion profile of differently primed HMDMs, SN of untreated HMDMs displayed the lowest CP-activating potential. The highest CP-activating capacity was observed testing C1q secreted by HMDMs incubated on imC1q+SLEIgG(1) as evident at every SN dilution. C1q secreted by cells exposed to imC1q alone exerted a CP-activating potential which was in general lower as compared to anti-C1q-primed HMDMs but higher as compared to untreated cells. The CP-activating potential of C1q produced by HMDMs was in general lower than that of purified serum-derived C1q, being in line with previous reports on macrophages and iDCs (26, 70).

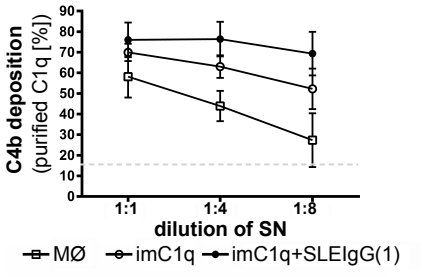


Figure 25: Activation of the CP by secreted C1q using an IgM-coated plate.

The ability of secreted C1q in activating the CP was investigated using an IgM-coated ELISA plate. HMDMs were left untreated or stimulated on imC1q or imC1q+SLEIgG(1) for 22h. SN of 6 different donors used to obtain HMDMs were pooled and processed as described in patients and methods. Serial dilutions of concentrated C1q-containing SN were diluted in 10% C1qDS/VBS and added to IgM-coated plates. The activation of the CP was assessed by detecting C4b deposition. Dashed grey line represents IgM-coated plates incubated with 10% C1qDS/VBS only. O.D. values were normalized to the corresponding O.D. values obtained when purified C1q was diluted in C1qDS/VBS at the same C1q concentration as measured in processed SN set to 100%. Data are shown as the mean ± SEM of 4 independent experiments using pooled SN of 6 different HMDMs donors.

Macrophages obtained from SLE patients also secrete C1q

So far, all experiments were performed using HMDMs obtained from healthy donors. Therefore, we next studied HMDMs derived from SLE patients at different stages of disease (Table 11) to assess whether differentiated macrophages of SLE patients show a similar C1q secretion profile as compared to healthy donor-derived cells.

Similar to healthy donor-derived cells, HMDMs obtained from SLE patients secreted higher C1q levels when these cells were incubated on imC1q as compared to those stimulated on HSA alone ($p<0.05$*) (Figure 26A). Providing a mild inflammatory stimulus by adding LPS to SLE HMDMs incubated on imC1q or HSA further increased C1q secretion levels (imC1q+LPS vs imC1q/HSA+LPS: $p<0.05$*). Importantly, SLE patient-derived HMDMs also strongly reacted to the combination of self-anti-C1q bound to imC1q (data not shown). This phenomenon could be observed in the majority of SLE patients and was dependent on anti-C1q serum levels (Table 11).

However, when SLE patient-derived HMDMs were incubated on anti-C1q bound to imC1q, the cells significantly enhanced their C1q secretion as compared to imC1q+NHIgG-incubated cells ($p<0.05$*) (Figure 26B).

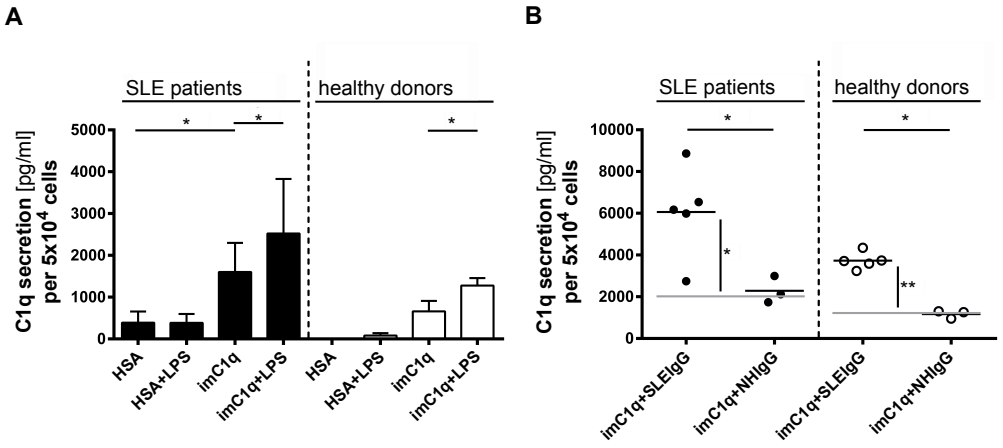


Figure 26: Macrophages of SLE patients show a similar C1q secretion profile.

CD14-positive monocytes obtained from SLE patients (n=6) or as control from healthy donors (n=5) were differentiated into macrophages and incubated on different coatings. After 22h, SN were collected and analyzed for secreted C1q levels. SLE patient-derived macrophages (black bars) and healthy donor-derived macrophages (white bars) were incubated on HSA or imC1q with or without LPS (A). Shown are secreted C1q levels of at least 5 different donors used to isolate cells. One-way ANOVA including Bonferroni's post-test, $p<0.05$*. SLE patient-derived macrophages (●) or healthy donor-derived macrophages (○) were incubated on imC1q+SLEIgG of unrelated anti-C1q positive SLE patients (n=5 different IgG preparations) or on imC1q+NHIgG (n=3 different IgG preparations) (B). Grey line represents mean C1q secretion levels of HMDMs incubated on imC1q alone. Each data point represents pooled C1q secretion levels (mean) of at least 5 independent experiments. Mann-Whitney U test, $p<0.05$*.

In general, when comparing C1q secretion levels of healthy donor-derived HMDMs (Figure 19) to SLE patient-derived HMDMs (Figure 26), we observed a trend towards an elevated C1q secretion capacity in SLE patient-derived cells for all stimulation conditions used, however, differences did not reach significance (Table 13).

Table 13: Comparison of C1q secretion levels of HMDMs obtained from healthy donors or SLE patients.

Stimulation condition	**C1q** [pg/ml] **by healthy donor-derived HMDMs** (n=5)	**C1q** [pg/ml] **by SLE-derived HMDMs** (n=6)	**p values** (Mann-Whitney U test)
HSA	60±39	390±265	p=0.09
HSA+LPS	82±59	384±213	p=0.31
imC1q	662±552	1'603±695	p=0.94
imC1q+LPS	1'278±180	2'524±1'304	p=0.94
imC1q+SLEIgG(1)	3'591±1'341	5'985±2'851	p=0.95
imC1q+NHIgG(1)	1'830±72	2'284±371	p=0.23

Shown are means ± SEM of 5 or 6 different donors.

In conclusion, SLE patient-derived HMDMs have a similar C1q secretion profile as compared to healthy donor-derived cells.

Discussion

Anti-C1q are believed to be pathogenic in lupus nephritis. This hypothesis is based on the presence of both, C1q and anti-C1q, in glomeruli of patients with proliferative lupus nephritis (11, 12) and anti-C1q were found to strongly correlate with the occurrence of lupus nephritis (7-10). However, their biological and pathogenic properties in this inflammatory kidney disease are not well defined. In parallel, anti-C1q seem to influence C1q levels, as anti-C1q negatively correlate with C1q serum levels (29, 183). This might be due to enhanced complement activation as demonstrated recently (183). However, anti-C1q might also be able to directly alter C1q secretion by HMDMs. In this study, we can now demonstrate that imC1q and to a higher extent anti-C1q bound to imC1q stimulate C1q synthesis and secretion by HMDMs. In addition, this secreted C1q is functional, i.e., able to activate the CP of complement. Thus, bound anti-C1q can induce a potent C1q-producing phenotype in macrophages. This enhanced C1q secretion might potentiate anti-C1q induced complement activation and the binding of additional anti-C1q molecules leading to a local vicious circle.

As opposed to most other complement proteins, C1q has a non-hepatic origin and is predominantly produced by myeloid cells, such as DCs and macrophages (26, 68-70). Therefore, free C1q is limited to the tissue due to local synthesis by these cells, whereas in the circulation C1q is mostly associated with its proteases C1s and C1r to form the C1 complex in the circulation (65, 66). *In vitro*, C1q synthesis can be influenced by different agents, including LPS, steroids, cytokines, and ICs (27, 69, 104, 119). Interestingly, in a previous report, imC1q triggered its own production by DCs but not by HMDMs (120). In contrast, Galvan et al. reported that imC1q upregulates C1q mRNA in murine macrophages (121). We found that both imC1q and even more imC1q-bound anti-C1q induce C1q secretion by HMDMs.

The additional effect of anti-C1q cannot be explained by the recruitment of FcγRs alone as using ICs or IgG-coating alone only induced low levels of secreted C1q. Therefore, we hypothesize that a double trigger of macrophages by imC1q via C1q receptors and bound anti-C1q via FcγRs is required for the observed C1q secretion profile. The pathway, however, leading to upregulated C1q synthesis and the regulation of the genes encoding C1q is not well understood. In general, it is believed that locally secreted C1q may act in an auto-/paracrine manner maintaining tissue homeostasis by suppressing cell-mediated inflammation. We observed that HMDMs on imC1q and bound-anti-C1q continuously secreted high C1q levels and produced an accumulation of iC1q suggesting continued *de novo* C1q synthesis, indicating that high levels of secreted C1q are unlikely to act as a negative feedback mechanism. Our data is in accordance with findings published by Zhou et al. who found that

murine macrophages continuously secreted C1q triggered by LPS, ICs, or C3b-opsonized zymosan (27). In contrast, C1q secretion by HMDMs remained unaffected when cells were incubated with IgG-coated sheep erythrocytes, yeast, or latex beads (69). Furthermore, addition of exogenous C1q to murine macrophages led to an increase in C1q mRNA levels (28). Taken together, these reports suggest that C1q secretion might act as a positive feedback, i.e., reflecting a temporal requirement of an increased amount of C1q in inflamed tissue (130, 131) and underlining the importance of local C1q production in the regulation of tissue homeostasis during inflammation.

To evaluate changes in the C1q secretion profile by macrophages at the site of inflammation, macrophages have been stimulated with different agents: Cytokines and different drugs have been tested in their properties to modulate C1q secretion. Whereas IL-1β and IFNγ decreased C1q biosynthesis over time, IL-6 acted as a potent stimulus for C1q secretion (119). Recently, we found that bound anti-C1q induce a pro-inflammatory phenotype in HMDMs (247). We observed that imC1q alone and bound anti-C1q induce C1q secretion by HMDMs. As it has been demonstrated that pro-inflammatory macrophages secrete considerably more C1q as compared to resident peritoneal macrophages (103, 248), our observation supports the view that C1q secretion is, in general, enhanced under inflammatory conditions.

A recent report indicated that infiltrating mononuclear cells play a role in the progression of lupus nephritis and are associated with poor prognosis in SLE patients (233). In addition, data from murine models suggest that macrophages and DCs infiltrating nephritic kidneys display an activated and heterogeneous phenotype and contribute to renal damage (193, 194). In kidney biopsies of lupus nephritis patients, immunoglobulins (IgG, IgA, IgM) are deposited close to complement proteins (C1q, MBL, C4, C3) (11, 12). Furthermore, concentrations of deposited anti-C1q in the glomeruli are up to 50times above those found in the sera of patients (223) leading to the conclusion that anti-C1q deposition and consecutive activation of infiltrating mononuclear cells occur in kidneys and contribute to the pathogenesis of lupus nephritis. Based on the fact that myeloid cells are a rich source for C1q, a common hypothesis is that macrophages and DCs contribute to high local C1q concentrations and consequently deposition in inflamed tissues. Indeed, it has been reported that C1q mRNA expression in kidneys was strongly upregulated *in vivo* in MRL/lpr mice suffering from severe lupus nephritis. Using this lupus-prone mouse model, infiltration of myeloid cells and local C1q production was associated with disease progression (252). Finally, MRL/lpr mice have elevated levels of anti-C1q present in serum as well as deposited in the kidneys which negatively correlate with low serum C1q levels (253).

Many SLE patients display hypocomplementemia during flares characterized by low or even undetectable C1q serum levels which negatively correlate with anti-C1q titers (7-9). Notably, Tan et al. did not find an association of C1q serum levels with glomerular C1q deposition in SLE patients. The authors concluded that serum C1q might contribute only little to C1q deposition in nephritic kidneys, whereas local C1q production by DCs and macrophages might be the main source of deposited C1q (254). At sites of local inflammation accompanied by increased apoptotic cell load, it is thought that locally secreted C1q has a major impact in the clearance of dying cells and has immune-regulatory functions in cell-mediated inflammation. Beyond complement activation, C1q plays a critical role in the clearance of apoptotic cells by bridging apoptotic cells and phagocytes thereby facilitating the uptake of apoptotic cells (22, 23, 120). Furthermore, during the phagocytosis of apoptotic cells, the production of C1q might be elevated due to an increased temporal requirement of C1q and consequently, facilitate the clearance of dying cells. Indeed, C3b-opsonized zymosan (resembling the surface of an apoptotic cell) increased C1q production by murine macrophages mainly through C3b receptor triggering (27). In addition, Galvan et al. reported that adhesion to C1q is followed by an upregulation of genes encoding C1q (121). Thus, enhanced C1q production might lead to an improved clearance of apoptotic cell material. However, this mechanism seems to be contradictory to the observation that anti-C1q decrease the phagocytic capacity of HMDMs and THP-1 cells (154, 247).

The defective clearance of apoptotic material by macrophages has been proposed as a mechanism underlying SLE pathogenesis causing an accumulation of dead cell material (20). It is well established that SLE patient-derived macrophages exhibit a defect in the clearance of apoptotic cell material correlating with low complement levels (17, 18). This impaired phagocytosis might be due to functional C1q deficiency induced by a high consumption rate of C1q or an insufficient production of C1q by myeloid cells. Our data now provide evidence that anti-C1q rather enhance C1q production by HMDMs, indicating that freshly available C1q is quickly consumed in affected tissues. This effect might be beneficial in the context of the removal of apoptotic cell debris, but it still might have disadvantage effects. Notably, elevated C1q secretion by HMDMs triggered by im-C1q bound anti-C1q might result in more available C1q which then can bind to tissue-deposited ICs and consequently lead to the activation of complement. Deposition of further C1q might serve as an additional target for anti-C1q leading to a local vicious cycle including complement activation and inflammation.

In conclusion, we can show that imC1q-bound anti-C1q induce a potent C1q-producing phenotype in HMDMs. This anti-C1q-induced C1q secretion by HMDMs might be an important

disease exacerbating factor in SLE patients. This observation provides new insights into pathogenic mechanisms of anti-C1q and their role in SLE.

Conclusions, ongoing projects, and outlook

Conclusions

In my thesis, I developed an *in vitro* model to study the pathogenic effects of systemic lupus erythematosus (SLE) patient-derived high-affinity autoantibodies directed against C1q (anti-C1q) on the activation of the complement system and their down-stream effects on macrophages. In conclusion, I can demonstrate that anti-C1q have proinflammatory effects that could be pathogenic (Figure 27). First, these autoantibodies amplify complement activation via both the classical and lectin pathways but not via the alternative pathway (see part 1, p. 29, (249)). Second, anti-C1q alter the phenotype of human monocyte-derived macrophages (HMDMs) by cross-linking of Fcgamma receptors (FcγRs) and activate HMDMs (S. Thanei et al. (submitted); see part 2, p. 46). These autoantibodies induce a proinflammatory cytokine response by upregulating IL-1β, IL-6, and TNFα secretion, downregulating the phagocytosis rate of apoptotic cells and Mer tyrosine kinase (MerTK) expression, and upregulating lipopolysaccharide (LPS)-induced CD80, CD274, and MHC class II expression. Third, immobilized C1q (imC1q)-bound anti-C1q induced C1q secretion by HMDMs (S. Thanei et al. (submitted); see part 3, p. 73).

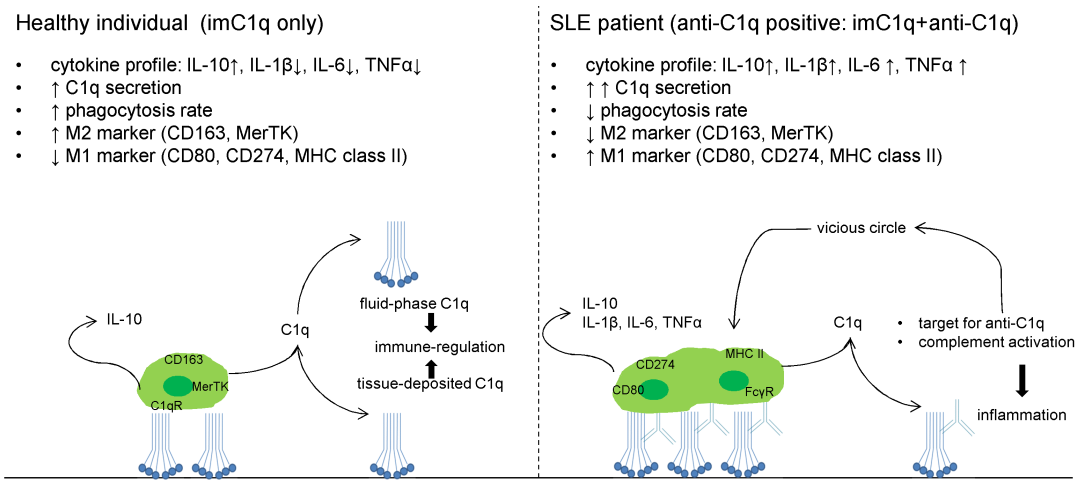


Figure 27: Overview of the functional consequences of the binding of SLE patient-derived high-affinity anti-C1q to imC1q.

The proposed effects of imC1q alone, as occurring in healthy individuals, and imC1q-bound anti-C1q, as occurring in SLE patients, are summarized.
Legend: C1qR: C1q receptor; M1: proinflammatory macrophages; M2: anti-inflammatory macrophages

Thus, anti-C1q have an effect on 2 major effector mechanisms of the immune system used by antibodies, the complement system as well as FcγRs. These interactions might play a critical role in lupus nephritis by the amplification of local inflammatory immune responses and consecutive tissue damage resulting in a local vicious cycle.

Ongoing projects and future perspectives

Ongoing projects and future perspectives are not included in the printed or online versions of this thesis.

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Curriculum vitae

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Academic Education

2012-present:	Graduate (PhD) studies in Immunology, Laboratory of Clinical Immunology, Department of Biomedicine Supervisor: Prof. Dr. M. Trendelenburg Thesis title: *Functional consequences of anti-C1q autoantibodies form systemic lupus erythematosus patients*
2011:	Swiss Federal Diploma for Pharmacists
2009-2011:	Master of Science in Pharmacy, University of Basel
2010:	Undergraduate studies in Pharmacy (Pharmacokinetics), Department of Pharmacy, College of Pharmacy, Gainesville, FL, USA Supervisor: Prof. Dr. V. Butterweck Master thesis title: *Pharmacokinetics of valerenic acid in rats after intravenous and oral administrations*
2005-2009:	Bachelor of Science in Pharmaceutical Sciences, University of Basel
2002-2005:	High school diploma in Oberwil (BL)

Working experience

2012-present:	PhD student at the Department of Biomedicine, Laboratory of Clinical Immunology, University Hospital Basel
2011-present:	Part-time pharmacist at “Steinen Apotheke”, Basel
2010-2011:	Trainee at “Steinen Apotheke”, Basel
2010:	Master student at the Department of Pharmacy, College of Pharmacy, Gainesville, FL, USA

Laboratory skills

- Cell culture: Isolation of monocytes and culture of HMDMs and DCs, cell isolation using magnetic-activated cell sorting (MACS); ELISA; ELISA development
- Complement activation assays
- IgG and protein purification methods: affinity chromatography (fast protein liquid chromatography (FPLC)); Protein G; ammonium sulfate precipitation method
- Flow cytometry: Cyan^{TM} ADP Analyzer (Dako, CA, USA), Accuri and LSR Fortessa (both instruments manufactured by BD Biosciences, CA, USA); data analysis using FlowJo Software (Tree Star Inc., OR, USA)
- ImageStream^{X} flow cytometry; data analysis using IDEAS software (Amnis, Seattle, WA)
- Microscopy: phase contrast microscopy, fluorescence microscopy; data analysis using ImageJ software
- Molecular biology and Biochemistry: gel electrophoresis; Western Blot

Languages

German (mother tongue); English (fluent); French (basic knowledge)

Publications

Sampath, C., Haug, K., **Thanei, S.**, Hamburger, M., Derendorf, H., Frye, R., Butterweck, V.: *Pharmacokinetics of valerenic acid in rats after intravenous and oral administrations.* Planta Med, 2012.

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Oral presentations

Thanei, S., Vanhecke, D., Trendelenburg, M.: *Functional consequences of SLE autoantibody binding on macrophages.* Immunomeeting, November 2013, Department of Biomedicine, University Hospital Basel, Basel.

Thanei, S., Trendelenburg, M.: *Anti-C1q autoantibodies from SLE patients induce a proinflammatory phenotype in macrophages.* European Meeting on Complement in Health and Disease, August 2015, Uppsala, Sweden.

Poster presentations

Vanhecke, D., **Thanei, S.**, Ploix, C., Trendelenburg, M.: *Complement activation: functional consequences of circulating anti-C1q autoantibodies and structural prerequisites for complement fixation by IgG in solution.* Roche Hub meeting, November 2012, Penzberg, Germany.

Thanei, S., Vanhecke, D., Ploix, C., Trendelenburg, M.: *Effect of anti-C1q antibodies from SLE patients on complement activation*. European Meeting on Complement in Health and Disease, August 2013, Jena, Germany.

Thanei, S., Vanhecke, D., Trendelenburg, M.: *Anti-C1q autoantibodies from SLE patients activate the complement system via both the classical and lectin pathways.* European Meeting on Complement in Health and Disease, June 2015, Uppsala, Sweden.