Immunological Consequences of C1q and Anti-C1q Immune Complexes in Secondary Cellular Inflammation

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List of Abbreviations

AA	amino acid
ACR	American College of Rheumatology
aFU	arbitrary fluorescence units
ANA	anti-nuclear antibodies
ANOVA	analysis of variance
anti-C1q	anti-C1q autoantibodies
anti-dsDNA	anti-double stranded DNA
AP	alkaline phosphatase
APCs	antigen-presenting cells
APC	allophycocyanin
AU	arbitrary units
BSA	bovine serum albumin
BV	Brilliant Violet™
C1q-biotin	biotin-labeled C1q
cC1q	collagen-like domain of C1q
cC1qR	cC1q receptor
CD	cluster of differentiation
CD40L	CD40 ligand
CFSE	carboxyfluorescein succinimidyl ester
CI	confidence interval
CLR	collagen-like region
CRISPR	clustered regularly interspaced short palindromic repeats
Cy	Cyanine
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DC-SIGN	DC-specific intracellular adhesion molecule 3-grabbing non-integrin
DMEM	Dulbecco's modified essential medium
DMSO	dimethyl sulfoxide
EBNA-1	EBV nuclear antigen 1
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia
ELISA	enzyme-linked immunosorbent assay
ERK	signal-regulated protein kinase
EULAR	European Alliance of Associations for Rheumatology
Fab	antigen-binding fragment
FACS	fluorescence-activated cell sorting
FB	factor B
Fc	fragment crystallizable
FD	factor D
FITC	fluorescein isothiocyanate

gC1q	globular heads domain of C1q
HBS	HEPES buffered saline
HEp-2	human epithelial type 2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMDMs	human monocyte-derived macrophages
HRP	horseradish peroxidase
HSA	human serum albumin
HUVS	hypocomplementemic urticarial vasculitis
i.e.	id est
IFNγ	interferon-γ
Ig	immunoglobulin
IL	interleukin
IQR	interquartile range
JAK	Janus family kinase
JNK	c-Jun N-terminal kinase
LAIR-1	leukocyte-associated immunoglobulin-like receptor 1
LN	lupus nephritis
LPS	lipopolysaccharide
MAC	membrane-attack complex
MASP	MBL-associated serine proteases
MBL	mannose-binding lectin
MFI	mean fluorescence intensity
MTT	tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	nuclear factor κB
NH	normal human
NHS	normal human serum
OD	optical density
OR	odds ratio
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PE	phycoerythrin
PGA	Physician's Global Assessment
qPCR	quantitative polymerase chain reaction
RD	rhabdomyosarcoma
ROC	receiver operating characteristic
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute medium
SELENA	Safety of Estrogens in Lupus Erythematosus National Assessment
SEM	standard error of the mean
SLE	Systemic lupus erythematosus
SLEDAI	SLE Disease Activity Index
SSCS	Swiss Systemic Lupus Erythematosus
TCR	T cell receptor
Tfh	T follicular helper cells

Th	T helper cell
TMB	3,3', 5,5' tetramethylbenzidine
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAF	tumor necrosis factor receptor-associated factors
Treg	T regulatory cell

Summary

The complement system comprises numerous plasma proteins that are finely orchestrated in a network of at least three separate pathways, namely the classical, lectin, and alternative pathway.[1] This system represents a cornerstone of the innate immune system and plays a crucial role in first-line defense against invading pathogens. Additionally, the complement system displays other functions, including self-tolerance.[2]

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease with heterogeneous clinical manifestations and pathogenic mechanisms that are not fully understood. Although genetic deficiencies in the early components of the classical pathway (i.e., C1q, C1r, C1s, C4, and C2) are rare in humans, they are strongly associated with the development of monogenic SLE, particularly hereditary homozygous C1q deficiency.[3–5] For instance, studies with mice deficient in C1q have strengthened this observation because affected animals developed an SLE-like disease.[6] However, primary deficiency for C1q is uncommon in humans.[5] Instead, most patients with SLE suffer from secondary hypocomplementemia associated with C1q autoantibodies (anti-C1q) present in 20–50% of patients.[7–9] Notably, anti-C1q appear to play a role particularly in lupus nephritis (LN) since individuals with renal involvement have displayed increasing levels of anti-C1q before exacerbation and pronounced anti-C1q deposition in the glomeruli.[10,11] However, exactly how anti-C1q contribute to disease activity and LN remains unclear.

Therefore, this dissertation investigates the immunological consequences of C1q and anti-C1q forming immune complexes in secondary cellular inflammation in the context of SLE and examines the following questions:

Part I – Do anti-C1q induce pro-inflammatory cytokine secretion in peripheral blood mononuclear cells (PBMCs) in the presence of activated T cells?

Although C1q alone has anti-inflammatory effects on human immune cells (i.e., monocytes, macrophages, dendritic cells [DC], and T cells)[12–15], our group previously demonstrated a pro-inflammatory phenotype in human monocyte-derived macrophages (HMDMs) induced by C1q/anti-C1q complexes *in vitro*.[16] However, the immunological consequences of C1q and C1q/anti-C1q complexes on other immune cells is unknown. Thus, in the first part of my thesis, I investigated the immunological effects of C1q and C1q/anti-C1q complexes in PBMCs with concomitant T cell activation. In an *in vitro* model for anti-C1q-mediated autoimmunity, I

demonstrated that C1q/anti-C1q complexes produced an upregulation of tumor necrosis factor (TNF), interleukin 10 (IL-10), and interferon-γ (IFNγ) secretion in PMBCs. Specifically, activated T cells elicited a cell–cell contact-mediated increase in TNF and IFNγ secretion in monocytes. Moreover, the co-stimulatory pair cluster of differentiation (CD)40–CD154 was essential for the release of TNF in C1q/anti-C1q-conditioned monocytes. The latter depended on the tumor necrosis factor receptor-associated factors (TRAF) 6 and Janus family kinase (JAK) 3-signal transducer and activator of transcription (STAT) 5 signaling pathways.

Part II – What are the phenotypical characteristics of low and high C1q-producing HMDMs and what are the autocrine and paracrine effects of de novo synthesized C1q?

Unlike most proteins in the complement system, the majority of C1q is of non-hepatic origin.[17] Instead, C1q synthesis predominantly occurs locally in tissue-resident myeloid cells, such as macrophages and DCs.[18–21] Regarding the regulation of C1q synthesis, our group previously reported a continued *de novo* synthesis of C1q in HMDMs, mediated by C1q and C1q/anti-C1q.[22] Although the overall concentration of C1q increased, not all HMDMs were equally involved in the production of new C1q. In the second part, I sought to define the heterogenous phenotypes of HMDMs and explore the potential autocrine and paracrine effects of the newly secreted C1q on immune cells. Combining transcriptional analysis of C1q mRNA and experiments inhibiting protein synthesis and secretion produced contradictory data that do not suggest *de novo* synthesis of C1q. Beyond this, coating of biotin-labeled C1q (C1q-biotin) revealed a considerable amount of the molecule in the cell culture medium to be derived from the plate. Together, the findings presented in this part of this thesis do not support the proposed notion of *de novo* synthesis of C1q in HMDMs triggered by C1q and C1q/anti-C1q complexes.[22]

Part III – Do epitope-specific anti-C1q associate with specific SLE disease manifestations?

Anti-C1q are high-affinity polyclonal autoantibodies that predominantly recognize epitopes located in the collagen-like region (CLR) of C1q.[23–26] There is weak to no binding of anti-C1q to soluble C1q, whereas attachment of C1q to a target allows anti-C1q to access neo-epitopes. However, the specificity of the autoantibodies has yet to be determined.

In the last part of this thesis, we explored epitopes of C1q and investigated whether epitope-specific anti-C1q were associated with specific clinical presentations. In the first step, we investigated the epitope-specificity of patient-derived anti-C1q using a high-resolution epitope mapping approach. By using peptide microarrays to map the epitopes of anti-C1q, we identified

three peptides of the C1q A-chain and three of the C1q B-chain with increased immunoglobulin (Ig) G binding. Next, screening a large SLE patient cohort by a newly established peptide-based enzyme-linked immunosorbent assay (ELISA) revealed that certain peptide-specific antibodies associated with selected disease manifestations. Notably, anti-C1q directed against the N-terminal C1q A-chain improved discrimination between controls and SLE beyond the conventional determination of anti-C1q.

Introduction

Complement System

First discovered in the 19th century, the term "complement" was coined by the German immunologist and Nobel laureate Paul Ehrlich in 1899.[27] The predecessors of the complement system date far back in evolution and are found in numerous lower species, primarily in charge of recognizing invading pathogens and mediating phagocytosis.[28] As evolution continued and more complex species with more developed immune defenses emerged, the complement system was not replaced but rather diversified with additional components and new effector functions, such as cell differentiation, chemotaxis, and bridging innate and adaptive immunity.[28,29] This and the fact that many of the genes of the complement system were preserved during evolution, sharing a high homology between species, highlights the pivotal role of complement in immunity and general physiology.[30]

The complement system comprises numerous plasma proteins and is divided into at least three separate pathways: the classical, lectin, and alternative pathways. These converge at the terminal pathway forming the membrane attack complex (depicted in Figure 1).[27,31] Beyond this, the classical pathway is initiated when the C1 complex ($C1qr_2s_2$) binds to a target structure (e.g., the membrane of an invading microbe) through its pattern recognition protein C1q, either directly or indirectly to the fragment crystallizable (Fc) region of multiple adjacent antibodies. Conformational changes in the CLR of C1q allow C1r to cleave C1s, which leads to cleavage of C4 and C2 into C4a and C4b, and C2a and C2b, respectively. Subsequently, C4b attaches to the cell membrane and joins C2a to form C4b2a (C3 convertase). The mannose-binding lectin (MBL) and ficolins represent the starting point for the lectin pathway. These proteins bind to specific carbohydrate structures on microbial surfaces and associate with two serine proteases, MBL-associated serine proteases (MASP) 1 and 2. Again, binding of the pattern recognition molecule causes conformational changes facilitating the activation of MASP-2, which can then cleave C4 and C2. Similar to the classical pathway, this reaction forms a C3 convertase. Lastly, the alternative pathway has two modes of activation. In the first mode, covalently bound C3b generated by any of the three complement pathways enables factor B to bind to C3b. In this confirmation, factor D can cleave factor B into Ba and Bb. Subsequently, the resulting product, Bb, joins with C3b to form C3bBb, the alternative C3 convertase. The other mode involves the spontaneous activation of C3 by hydrolysis. Similar to the first mode of the alternative pathway, factors B and D form $C3(H_2O)Bb$, a short-lived fluid-phase C3 convertase. At this point, all three cascades have

generated C3 convertases, which ultimately produce C3b and complete the C5 convertase (C3b2b3b or C3bBb3b). The generation and deposition of C5b by the C5 convertase triggers the recruitment of the terminal components C6 to C9. Finally, the polymerization of C9 completes the pore-forming membrane-attack complex (MAC) which lyses the target structure, such as invading cells.

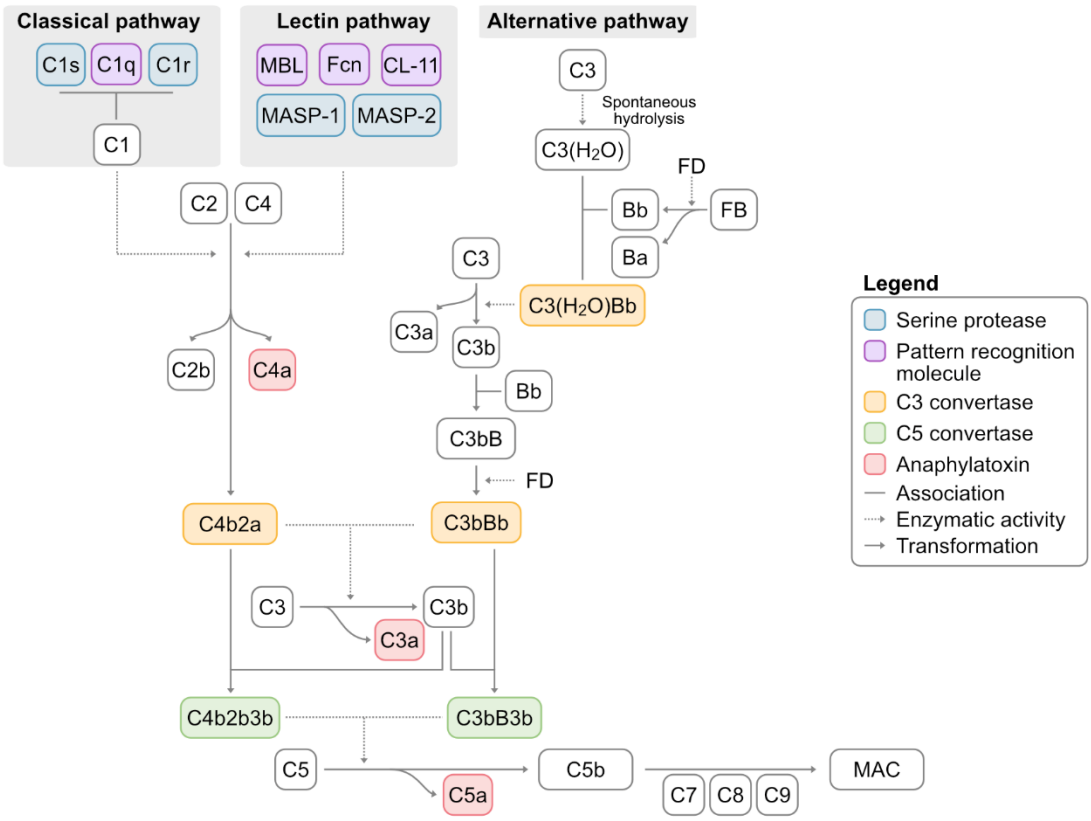


Figure 1 | Diagram of the complement system

The complement system consists of the classical, lectin, and alternative pathways with various components involved. All three pathways lead to the formation of C3 convertase and the terminal formation of the membrane-attack complex (**MAC**).

Abbreviations - CL-11: collectin 11, FB: factor B, Fcn: ficolin, FD: factor D, MASP: MBL-associated serine protease, MBL: mannose-binding lectin.

In addition to all the proteins of the complement system involved in the formation of the MAC, C3a, C4a, and C5a act as potent anaphylatoxins.[31] According to their names, these molecules mediate local immune responses through vasodilation, enhanced vascular permeability, chemotaxis, and the generation of reactive oxygen species.[27,31]

Complement C1q

The initiator molecule of the classical pathway, C1q is a 460 kDa glycoprotein with a hexametric quaternary structure resembling a bouquet. This entire protein comprises 18 polypeptide chains of three different types named C1qA, C1qB, and C1qC, encoded by three separate synchronously transcribed genes *C1QA*, *C1QB*, and *C1QC* all located within the region 1p34.1–1p36.3 of chromosome 1.[32–35] The first levels of arrangement are A–B and C–C chain dimers formed by disulfide bridges at the N-terminal ends.[36] Next, two A–B dimers and one C–C dimer build a heterotrimeric subunit, of which a total of three subunits form C1q (Figure 2). The N-terminus of all polypeptide chains contains a collagen-like sequence (-Hyp-Gly-Lys-Xaa-Gly-Pro-)[37] mediating immune effector mechanisms, followed by a hinge and a globular head module at the C-terminus, responsible for pattern recognition.[38]

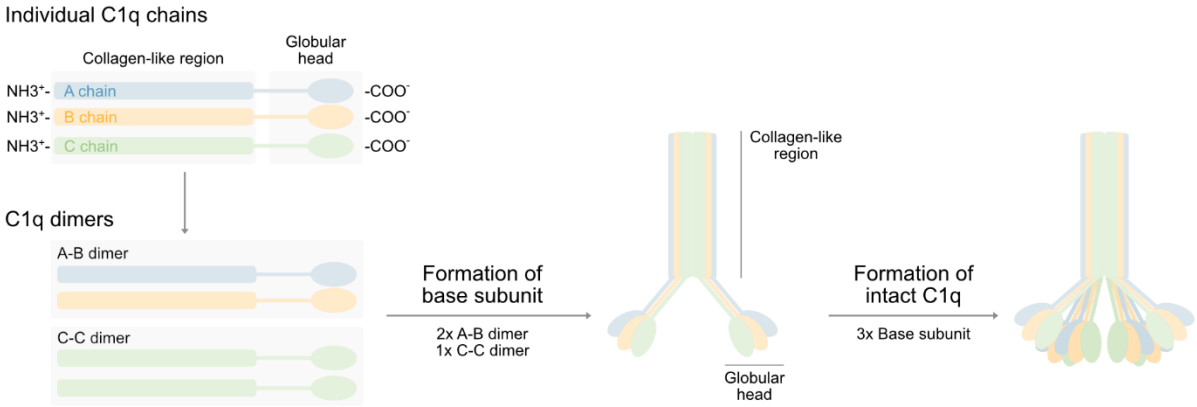


Figure 2 | Schematic representation of C1q

The final protein requires the orchestrated assembly of 18 polypeptide chains (six C1q-A, six C1q-B, and six C1q-C) over multiple steps with the CLR and the globular heads located at the N-terminus and C-terminus, respectively. First, the assembly involves dimerization of single polypeptide chains resulting in A–B and C–C dimers. Next, two A–B dimers associate with a C–C dimer to create a heterotrimeric base subunit. Ultimately, a total of three base subunits form the glycoprotein C1q.

One of the primary functions of C1q is to initiate the cascade of the classical pathway as part of the C1 complex ($C1qr_2s_2$). First, C1q, the recognition protein of C1, binds to the Fc region of antigen-bound Igs (Ig; IgM and IgG1, IgG2 and IgG3 isotypes).[39] This process induces a conformational change in the CLR allowing C1s and C1r, both serine proteases, to induce the activation of downstream enzyme complexes.[31]

In addition to complement activation, C1q was shown to elicit numerous complement-independent functions. Detached from the C1 complex, C1q is considered an opsonin binding a

multitude of target structures (e.g., apoptotic cells, pentraxins, lipid A, and β-sheet amyloid fibrils)[38] through its globular head module. Additionally, the binding of the CLR to C1q receptors (e.g., cC1qR, gC1qR, and C1qRp) mediates phagocytosis by macrophages and DCs.[38,40,41] Furthermore, C1q exerts chemotactic and immunomodulatory properties in DCs and macrophages by engaging DC-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and leukocyte-associated Ig-like receptor 1 (LAIR-1), creating an anti-inflammatory phenotype.[42–44] More non-conventional physiologic functions include tissue repair, induction of angiogenesis, embryo implantation, and synaptic pruning.[38,45,46]

Beyond C1q's physiological functions, it has been extensively studied in numerous diseases. For instance, C1q serum concentration can be used as a biomarker because it changes in some diseases.[47] Generally, low levels of C1q exist in several autoimmune conditions (e.g., SLE, hypocomplementemic urticarial vasculitis syndrome [HUVS], and rheumatoid arthritis) as a result of high complement consumption. On the other hand, increased levels of C1q often result from infectious diseases, inflammatory diseases (e.g., chronic obstructive pulmonary diseases, chronic liver disease), neurological diseases (e.g., epilepsy, Alzheimer's disease, Parkinson's disease) and some cancers (e.g., breast cancer, renal cell carcinoma, and glioblastoma). However, the pathophysiologic mechanisms involved in each disease are largely unknown because C1q can have both beneficial and detrimental effects depending on the disease. Recently, Schulz & Trendelenburg reviewed numerous studies exploring *C1qa*$^{-/-}$ mice to illustrate the potential pathophysiologic contributions of C1q in specific diseases.[48]

The physiological concentration of C1q in healthy human blood ranges from 37 to 189 μg mL^{-1}.[49] Although, most circulating C1q is bound in C1 complexes, a small proportion of C1q is not bound in circulating blood or tissue.[50] Although plasma C1q can be synthesized by Kupffer cells within the liver, contrary to most proteins involved in the complement cascades the main source of C1q is extrahepatic.[17] However, C1q present in tissue is widely considered to be synthesized and secreted locally by cells in tissues where C1q regulates a wide range of immunological processes in both autocrine and paracrine manners.[17] Many of these cells are ubiquitously distributed throughout the body and belong to the myeloid lineage, including monocytes, macrophages (i.e., microglia, osteoclasts, alveolar macrophages, Kupffer cells), DCs, mast cells, and non-myeloid cells such as mesenchymal cells, fibroblasts, and trophoblasts. Investigations of C1q biosynthesis by Michael Loos *et al.* revealed that C1q is synthesized as a transmembrane protein and firmly remains within the membrane, facing the extracellular space.[51–

[53] To release the protein into the microenvironment, the membrane-anchored form of C1q is enzymatically cleaved from the membrane rather than secreted by exocytosis.

The size of C1q allows multiple receptors present on the effector cell surface to bind and interact with the molecule. In past decades, numerous receptors in various cells have been described as making C1q responsible for its cellular functions.[29,38] Broadly, the receptors are divided into two groups, those binding either the CLR or the globular head domain (gC1q). For instance, the clearance of immune complexes and apoptotic cells is mostly mediated by cC1q receptors, such as cC1qR (calreticulin), complement receptor 1, low-density lipoprotein receptor-related protein 1 (CD91), and complement receptor 3 (CD11bCD18). On the other hand, differentiation of myeloid cells was shown to be reliant on gC1q receptors, including gC1qR, LAIR-1 (CD305), and DC-SIGN (reviewed by Thielens *et al.* and Reis *et al.*).[29,38]

Most of C1q's cellular immunomodulatory effects have been attributed to innate immune cells (e.g., DCs, monocytes, and macrophages), but an increasing number of publications have suggested that C1q also affects adaptive immunity, particularly T cells (Table 1 summarizes publications focusing on human primary peripheral T cells).

Early observations of C1q interacting with T cells led to the characterization of C1q-binding sites by several independent groups.[13,14,54,55] The immunomodulatory effects of C1q in T cells are classified as direct and indirect. Additionally, directly mediated immunomodulatory effects occur through direct C1q-binding to T cells, whereas indirect effects are mediated by other cells (e.g., macrophages and DCs), which are conditioned in the presence of C1q. In multiple studies, the direct binding of soluble C1q to T cells reduced the activation (e.g., CD25 and CD69), proliferation, and secretion of pro-inflammatory cytokines (e.g., IFNγ, IL-10, IL-4).[13,15,56,57] However, immune complexes containing C1q had contrary effects to soluble C1q, eliciting TNF and IFNγ secretion and expressing CD25.[58,59] Notably, direct C1q receptor-mediated immunomodulatory effects in T cells are not limited to C1q but also apply to related endogenous molecules (e.g., mannan-binding lectin and collectin 11) and viral proteins. For instance, proteins of the hepatitis B and C viruses (viral e antigen and core protein)[60–62] and human immunodeficiency virus (glycoprotein 41)[63,64] exploit C1q receptors to inhibit and escape the T cell response. This process further emphasis the immunomodulatory potential of C1q (receptors) in T cells. Regarding the potential indirect immunomodulatory effects of C1q in T cells, mixed lymphocyte reaction, an autologous or allogenic co-culture of T cells with either macrophages or DCs, revealed that T helper (Th)17 and Th1 cell proliferation was diminished with prior exposure of the phagocytes to C1q (bound to the cell culture plate or late apoptotic cells).[65,66] Thus, co-stimulatory

proteins (e.g., programmed death-ligand 1, CD40, CD80, and CD 86) on phagocytes influencing T cell activation are differentially expressed in the presence of C1q.

Table 1 | Selection of publications describing the direct and indirect immunomodulatory effects of C1q in T cells

Direct effects		
Cells	Experimental setup and main outcome in the presence of C1q	Ref
Primary peripheral T cells MOLT-4 cell line	Characterization of C1q–T cell interaction	54,55
Primary peripheral T cells	Addition of 50 μg mL^{-1} soluble C1q to cell culture - Characterization of C1q–T cell interaction - Reduced mitogen-induced proliferation	13
Primary peripheral T cells	Addition of 30 μg mL^{-1} soluble C1q to cell culture - Characterization of C1q–T cell interaction	14
Primary peripheral T cells	Addition of 50 μg mL^{-1} soluble C1q to cell culture or pre-coating with C1q (effect of bound-C1q) - Regulation of IFNγ, IL-10, and IL-4	15
Primary peripheral T cells	Addition of C1q-bearing ICs to the cell culture - Dose-dependent induction of TNF and IFNγ secretion - CD25 induction comparable to mitogen activation Cross-linking of surface-bound C1q by anti-C1q - Induction of TNF secretion	58,59
Primary peripheral T cells	Addition of 20 μg mL^{-1} soluble C1q to anti-CD3/CD28 (plate-bound) activated T cell culture - Reduced T cell proliferation ($CD3^+$, $CD4^+$, and $CD8^+$) - Calreticulin responsible receptor and inhibition of proximal TCR signaling	56
Primary peripheral T cells in PBMCs	Addition of 50 μg mL^{-1} soluble C1q to anti-CD3/CD28 (bead-bound) activated T cells - Reduced T cell proliferation ($CD3^+CD8^+$) - Reduced T cell activation CD25 and CD69 ($CD3^+CD8^+$) - Decreased effector function in $CD8^+$ T cells (granzyme B and IFNγ)	57
Indirect effects		
Cells	Experimental setup and main outcome in presence of C1q	Ref
Primary peripheral T cells and HMDMs	Autologous and allogenic T cell–HMDM (primed in the presence of LAL ± C1q) co-culture - Reduced Th17 and Th1 subset proliferation and slightly increased Treg proliferation	65
Primary peripheral T cells and DCs	Allogenic T cell–DC (primed ± bound-C1q) - Reduced Th17 and Th 1 induction - Reduced CD25 induction in $CD4^+$ cells after stimulation of DCs with LPS or IFNγ	66

Abbreviations – DC: dendritic cell, HMDM: human monocyte-derived macrophage, ICs: immune complexes, IFNγ: interferon-γ, IL: interleukin, LAL: late apoptotic cell, LPS: lipopolysaccharide, PBMCs: peripheral blood mononuclear cells, TCR: T cell receptor, Th: T helper cell, TNF: tumor necrosis factor, Treg: T regulatory cell

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disorder that affects multiple organs (e.g., skin, joints, central nervous system, kidneys). The diagnosis of SLE is challenging considering the heterogeneity of clinical presentation. Additionally, SLE's prevalence in Europe is estimated to be 28–97 cases per 100,000 citizens.[67,68] Beyond this, women of childbearing age diagnosed with the condition six to 15 times more often than men, depending on additional factors such as age, geographic region, and ethnic group.[68,69]

Currently, diagnosis of SLE is currently based on the 2019 European Alliance of Associations for Rheumatology (EULAR)/American College of Rheumatology (ACR) criteria, whereby clinical manifestations and laboratory test results are scored (Table 2).[70] To diagnose SLE, a positive test result for anti-nuclear antibodies (ANA), a total score of at least 10 points and at least one clinical manifestation are required. Disease activity changes over time and can be classified as active (a flare or relapse) or inactive (with residual low disease activity or complete remission).[71] To treat patients with SLE, the SLE Disease Activity Index (SLEDAI) facilitates assessment of the current severity of the disease and the need for medication.[72]

Generally, multiple intrinsic (genetic) and extrinsic (environmental) factors are considered in the pathogenesis of SLE.[73] The importance of extrinsic factors, such as UV light, drugs (e.g., isoniazid, hydralazine, procainamide), and smoking, is indicated by the fact that only one third of monozygotic twins share SLE.[73,74] Although SLE is most often polygenic, approximately 34 genes are known to cause monogenic SLE (reviewed in Alperin *et al.*).[75] These genes include some of the early components of the classical complement cascade (e.g., *C1QA/B/C*, *C1S*, *C3*, *C4A/B*), highlighting the importance of complement in the pathogenesis of SLE. Notably, homozygous C1q deficiency is one of the strongest genetic risk factors in disease development suggesting its crucial role in SLE.[3,5] A possible but not exclusive explanation is the "waste disposal hypothesis", as C1q-deficient individuals are known to have impaired clearance of apoptotic cells and immune complexes potentially causing an autoimmune response.[4,76] However, homozygous C1q deficiency is rare (77 case reports as of 2016)[77] and most patients suffer from secondary hypocomplementemia likely caused by increased complement activation via the classical and lectin pathways in association with the occurrence of anti-C1q antibodies (anti-C1q).

Table 2 | Classification criteria for SLE according to the 2019 EULAR / ACR

Entry criterion			
ANA titer of ≥ 1:80 on HEp-2 cells or an equivalent positive test (ever)†			
Additive criteria			
- Do not count a criterion if there is a more likely explanation than SLE - Occurrence of a criterion on ≥ 1 occasion is sufficient - Criteria need not occur simultaneously - Within each domain, only the highest weighted criterion is counted toward the total score if more than one is present§			
Clinical domains and criteria	**Weight**	**Immunology domains and criteria**	**Weight**
Constitutional - Fever	2	*Antiphospholipid antibodies* - Anti-cardiolipin antibodies OR - Anti- β2GP1 antibodies OR	2
Hematologic - Leukopenia - Thrombocytopenia	3 4	*Complement proteins* - Low C3 OR low C4 - Low C3 AND low C4	3 4
Neuropsychiatric - Delirium - Psychosis - Seizure	2 3 5	*SLE-specific antibodies* - Anti-dsDNA antibody* OR - Anti-Smith antibody	6
Mucocutaneous - Non-scarring alopecia - Oral ulcers - Subacute cutaneous OR discoid lupus - Acute cutaneous lupus	2 2 4 6		
Serosal - Pleural or pericardial effusion - Acute pericarditis	5 6		
Renal - Proteinuria > 0.5 g d^{-1} - Renal biopsy Class II or V LN - Renal biopsy Class III or IV LN	4 8 10		
If entry criterion fulfilled, a total score of 10 or more are required to classify as SLE			

†If ANA are absent, do not classify as SLE. §Additional criteria within the same domain will not be counted. *In an assay with 90% against relevant disease controls.

Abbreviations – ANA: anti-nuclear antibodies, Anti-β2GP1: anti-β2 glycoprotein 1 antibody, Anti-dsDNA: anti-double stranded DNA antibody, HEp-2: human epithelial type 2, LN: lupus nephritis, SLE: systemic lupus erythematosus.

Table modified from Aringer *et al.*[70]

In summary, the hyperactivity of autoreactive B cells and the presence of autoantibodies against intracellular components (e.g., nuclear and cytosolic antigens) and plasma proteins (e.g., C1q, albumin[78], prothrombin[79], and β2-glycoprotein 1 [Ref 80]), aberrant clearance of apoptotic cells, formation of immune complexes, and hypocomplementemia are some of the highly characteristic traits of SLE. Despite significant advances in recent decades that have raised the SLE patient survival rate, the treatment of patients with SLE still poses a formidable challenge with unmet needs.

Anti-C1q in SLE

Reduced complement levels in patients with SLE are a common feature of increased disease activity (item of the SLEDAI).[72] Hypocomplementemia is widely considered to be a consequence of increased complement activity rather than the primary deficiency of complement components or the presence of depleting autoantibodies.[81–84] Anti-C1q are non-depleting polyclonal autoantibodies with a high affinity for epitopes expressed in the CLR of C1q and most often belong to the IgG1 and IgG2 subclass.[23–26,85] Notably, these epitopes are not accessible by anti-C1q on fluid-phase (unbound) C1q. Prior, attachment of C1q to a target structure induces conformational changes in the CLR that exposes specific epitopes thus allowing anti-C1q to bind through their antigen-binding fragment (Fab).[86] These types of epitopes are called "neo-epitopes" or "cryptic-epitopes".

Anti-C1q are not unique to patients with SLE and are not considered an applicable biomarker for diagnosis of SLE. Among SLE, other autoimmune diseases and renal diseases (e.g., HUVS, rheumatoid vasculitis, mixed connective tissue disease, autoimmune thyroid disease)[87–89] as well as healthy individuals can develop detectable anti-C1q titers. In randomly selected healthy individuals, 11% of young adults (20–39 years) and 18% of elderly adults (70–79 years) displayed elevated levels of anti-C1q.[90] Nonetheless, anti-C1q has been widely adopted as a marker of disease activity in SLE. Considering the high negative predictive value of anti-C1q for proliferative LN, anti-C1q are suggested to be a necessary but insufficient element for renal involvement in SLE.[91–94] Furthermore, the localization of C1q within the renal subendothelial space and glomerular basement membrane is a specific histological finding in proliferative LN biopsies.[11,95,96] Beyond this, anti-C1q's accumulation in the glomeruli (50-fold higher concentrations than that in serum) and co-localization with C1q within electron-dense deposits further highlights the contributions of C1q/anti-C1q complexes in the pathogenic mechanism of proliferative LN.[10,11] Trouw *et al.* posed one possible explanation for the pathogenicity of anti-C1q in a study on anti-C1q.[97] These

researchers demonstrated that injecting a monoclonal mouse anti-mouse C1q antibody (JL-1, recognizing the CLR) in healthy C57BL/6 mice caused a subclinical renal disease with C1q and anti-C1q deposition, and mild granulocyte influx in the glomeruli of the animals. After the administration of polyclonal C1q-fixing anti-glomerular basement membrane antibodies, the exacerbation of previous renal damage with histological changes, increased granulocyte influx, and albuminuria was observed in the animals. The *in vivo* model demonstrated the potential pathogenicity of anti-C1q combined with deposited immune complexes, but this model did not fully reflect the situation in human patients with SLE. First, anti-C1q in humans are unlikely to be monoclonal, and second, JL-1 depleted serum C1q in mice. These two facts complicate the interpretation of the observations made by the authors and their translation into humans, thus necessitating further investigations.

The ability of C1q to exert its function is likely impeded by anti-C1q binding. Given the polyclonal nature of anti-C1q, it is likely that the degree of this impediment may depend on both anti-C1q levels and the specificity of the polyclonal anti-C1q. In an epitope mapping approach by Vanhecke *et al.*, a major linear epitope of C1q was discovered within the CLR, which was named "A08" at the time.[98] This epitope contains 13 amino acids (AA) and is located at position 15 on the C1qA-chain (AA sequence GRPGRRGRPGLKG). Furthermore, the authors reported a higher specificity for the "A08"-peptide ELISA than the traditional anti-C1q ELISA, improving discrimination between healthy individuals and patients with SLE. Notably, disease activity and prognosis, especially renal involvement, correlated better with "A08"-specific antibodies than with antibodies against intact C1q.[99]

How C1q becomes antigenic and causes the development of anti-C1q is not yet fully understood. Generally, autoantibodies are typically directed against complexes of proteins, nucleic acids, or both rather than against isolated structures.[86] For example, ANA target nuclear autoantigens aggregated in large protein–nucleic acid complexes that can be processed by professional antigen-presenting cells (APCs, e.g. macrophages, DCs, and B cells). The generation of anti-C1q in patients with SLE may be caused by the impaired clearance of apoptotic cells, a constant physiological process in the human body, and by the subsequent prolonged presence of attached C1q on apoptotic cells.[100] Additionally, Schaller *et al.* suggested that anti-C1q arise from an antigen-driven and antigen-matured immune response.[101] Additionally, the nuclear antigen 1 of the Epstein-Barr virus (EBNA-1), frequently suspected as a possible extrinsic factor for autoimmune conditions (such as SLE or multiple sclerosis), and the epitope "A08" share an eminent AA sequence homology. Specifically, an EBNA-1-derived peptide was capable of

inducing the production of anti-C1q antibodies recognizing C1q, particularly the "A08" epitope, in *C1qa*$^{-/-}$ mice.[102]

In light of these findings, it is reasonable to assume that anti-C1q result from a genetic predisposition for a determined response toward autoantibody formation as well as impaired clearance of apoptotic cells and immune complexes.

Macrophages in SLE

Analyses of circulating and tissue-resident macrophages derived from SLE patients and animal models revealed numerous aberrations in the activation and differentiation status as well secretory and effector functions (e.g., phagocytosis).[103] Strikingly, the ability to clear apoptotic cells and cell debris is diminished in macrophages of SLE patients which is associated with prolonged exposure of potential self-antigens and the formation of autoreactive antibodies.[104,105]

Furthermore, differentiation of macrophages in SLE patients showed an imbalance in M1-like and M2-like macrophages with increased signal transducer and activator of transcription (STAT) 1 and suppressor of cytokine signaling 3, and decreased STAT3, STAT6, and CD163 expression, respectively, thus favoring polarization towards a pro-inflammatory M1-like phenotype.[106] Interestingly, a mouse study revealed that transfer of M1-like macrophages deteriorated disease severity, whereas transfer of M2-like macrophages ameliorated the disease, indicating that M1-like and M2-like macrophages contribute to tissue damage and tissue repair in the kidneys of the mice, respectively.[107]

Taken together, aberrant clearance of apoptotic cells and an imbalanced M1- and M2-like differentiation highlight the involvement of macrophages in the development and maintenance of SLE.

T cells in SLE

T cells have been implicated to play an important role in the development of SLE. They are a complex group of highly differentiated immune cells divided into subpopulations, including effector, memory, and regulatory T cells.[27] In particular, the secretion of pro-inflammatory cytokines and helping B cells generate autoantibodies are two main contributing factors for T cells in maintaining the disease and causing tissue damage.[108,109] These aberrant processes are partially linked to abnormal T cell activation through a reduced TCR activation threshold and reduced peripheral tolerance in patients with SLE.[110]

In SLE patients, ratios of peripheral T cell subpopulations vary widely with an imbalance toward pro-inflammatory phenotypes. Notably, Th17 and their potent pro-inflammatory cytokine IL-17 are increased in SLE and in particular in LN.[111,112] On the other hand, regulatory T cells (Treg) responsible for controlling immune responses are reduced in SLE. This change in ratio between Th17 and Tregs could be caused by an insufficient IL-2 production, required to inhibit Th17 differentiation and maintain Tregs.[113,114] Additionally, C1q has been shown to indirectly influence the balance between Th17 and Tregs through phagocytes, favoring Tregs.[65,66]

Furthermore, the participation of T follicular helper cells (Tfh) in the generation of high affinity autoantibodies (e.g. anti-dsDNA) in SLE is highlighted by the IgG isotype and the somatic hypermutations.[108] These two characteristics indicate that B cell activation and production of antibodies occurs in a T cell dependent-manner. Notably, in the peripheral blood of patients with SLE T cells with a Tfh-like phenotype (characterized by C-X-C chemokine receptor 5) are significantly increased and their numbers correlate with the occurrence of anti-dsDNA.[115–118] Additionally, production of autoantibodies was shown to involve the co-stimulatory ligand CD154, found on activated T cells.[116]

Co-stimulatory molecule CD40

Besides the complement system, there are other important players of the immune system potentially involved in the development of SLE. One of them is the co-stimulatory molecule CD40 that mediates T cell-dependent B cell responses and is a likely candidate to play a role in autoimmune diseases.[119]

The adaptive immune system requires at least two signals for initiation. The first signal in T and B cells comes from the engagement of the antigen-specific T cell receptor (TCR) with the antigen on the major histocompatibility complex II presented by APCs and the B cell receptor directly binding to the native antigen, respectively.[27] Co-stimulatory molecules consist of receptor and ligand pairs, which deliver the necessary second signal in the initiation step between T cells and APCs, and between T cells and B cells. Additionally, co-stimulatory molecules can be divided into the CD28–B7 and tumor necrosis factor receptor (TNFR) families. The co-stimulatory molecule CD40 belongs to the TNFR and binds its ligand CD154, also called CD40 ligand (CD40L), present on activated T cells.[120] This co-stimulatory pair initiates two-way signaling transducing activating signals to the T cell and inducing the expression of B7 in DCs further promoting T cell proliferation.[27]

Originally discovered in B cells, CD40 is a 48 kDa type I transmembrane glycoprotein receptor consisting of 197 AA found in DCs, monocytes, macrophages, platelets, and other non-hematopoietic cells (e.g., myofibroblasts, epithelial, and endothelial cells)[121]. CD40's ligand CD154 is primarily expressed in activated T cells and exists as a soluble and type II transmembrane protein with a mass of 32–39 kDa with the ability to form homotrimers.[121] The CD40–CD154 coupling was initially described as involved in T cell-dependent B cell activation, differentiation, formation of germinal centers, and isotype switching.[122,123] Generally, CD40 signaling has a profound impact on cell survival, cytokine secretion, the expression of co-stimulatory surface markers, cross-presentation of antigens, and the differentiation of many cell types.[120] For instance, CD40 signaling in macrophages mediates the production of TNF and IL-1, both involved in local tissue destruction and chronic inflammation.[124–126]

The absence of intrinsic kinase activity in the cytosolic tail of CD40 requires the recruitment of adapter proteins belonging to the TRAF family.[127] Six members comprise the TRAF family, with TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 involved in intracellular CD40 signaling.[120,127] The release of the signaling complex to the cytosol allows activation of proximal transducing components phosphatidylinositol 3-kinase, nuclear factor κB (NFκB), and mitogen-activated protein kinases, such as extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and mitogen-activated protein kinase p38.[120] Additionally, the direct binding of JAK3 to the cytosolic tail of CD40 and subsequent phosphorylation of signal transducer and activator of transcription 5 (STAT5) allows TRAF-independent signaling.[128,129] Figure 3 illustrates intracellular CD40 signaling (as reviewed by Elgueta *et al.*[120]). Regarding pro-inflammatory cytokine production, TRAF6 proved to be an essential CD40 signaling component in human monocytes and macrophages because the presence of a TRAF6-binding peptide abolished TNF, IL-6, and IL-1β secretion.[130] Furthermore, the administration of a JAK3-specific small molecule inhibitor significantly improved disease activity in a model for rheumatoid arthritis in mice with low TNF, IL-6, and IL-1β serum concentrations.[131]

The CD40–CD154 interaction has been implicated in several cancer and autoimmune conditions, where aberrant expression of either CD40 or CD154 has been observed.[119,132] In SLE, patients with active disease displayed overexpression of CD154 in $CD4^+$ and $CD8^+$ T cells and ectopic expression in monocytes, B cells, and renal mesangial cells causing spontaneous antibody production and the release of transforming growth factor β (TGF-β), respectively.[133,134] One possible explanation for the importance of CD40 signaling in SLE and autoimmunity could be the following: i) the altered selection of T cells in the thymus (potential autoreactive clones escaping

deletion),[135] ii) the priming of T cells toward pro-inflammatory Th17 cells by B cells and APCs in secondary lymphoid organs,[136] or iii) the production of pro-inflammatory cytokines at the site of the tissue damage.[133,137] Both CD40 and CD154 represent potential targets for therapeutic interventions. For instance, the administration of the humanized monoclonal anti-CD154 antibody BG9588 to patients with proliferative LN in clinical trials alleviated symptoms by reducing anti-double stranded DNA antibodies (anti-dsDNA), increasing complement C3, and improving renal function. However, severe thromboembolic events discontinued the development.[138] Furthermore, the CD40 targeting antibody CFZ533, a humanized non-depleting anti-CD40 monoclonal antibody, inhibits the CD40-mediated activation of leukocytes *in vitro* and blocks both primary and T cell-dependent antibody responses *in vivo*. This antibody candidate did not produce any thromboembolic events due to a N297A mutation introduced in the Fc region.[139]

Although activation of T and B cells can occur without CD40–CD154 signaling, improper signaling through absence, overexpression, or ectopic expression of CD40 and CD154 can cause issues in mounting an appropriate adaptive immune response. The latter process has been observed in several cancer and autoimmune conditions.[120]

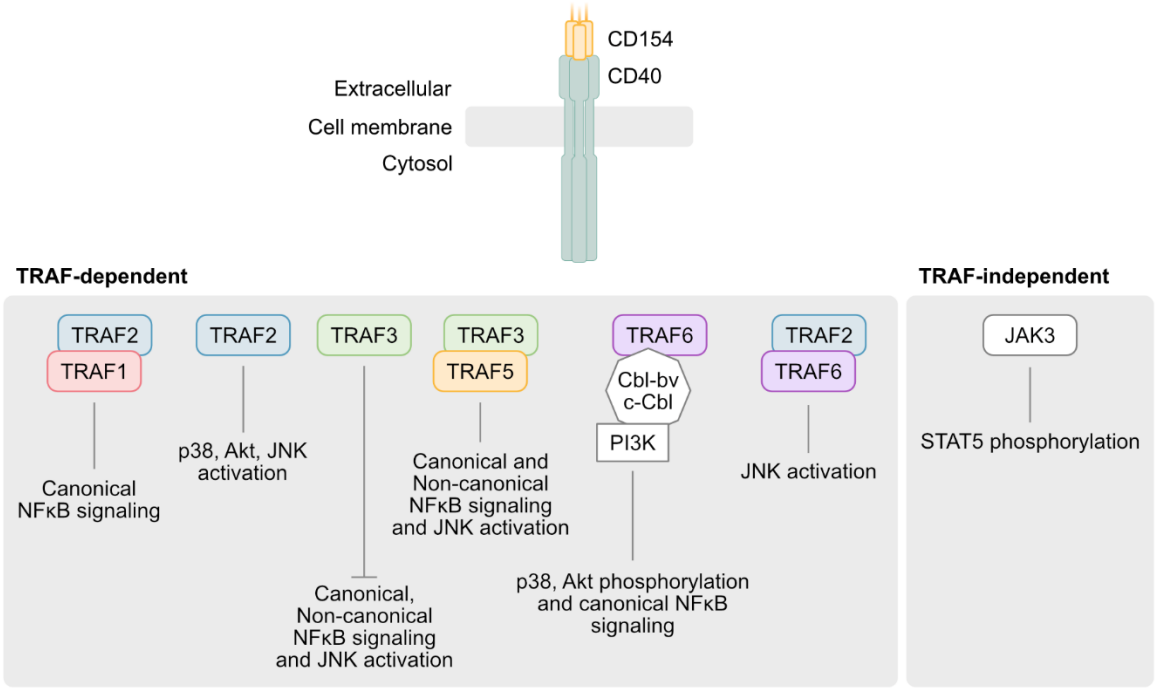


Figure 3 | Overview of intracellular CD40 signaling

Ligation of CD40 and CD154 initiates TRAF-dependent and TRAF-independent CD40 signaling. Additionally, TRAF1, 2, 3, 5, and 6 associate with the cytosolic tail of CD40 to form signaling complexes to further activate proximal transducing components. Next, TRAF-independent signaling occurs through the recruitment of JAK3 and the downstream phosphorylation of STAT5. Figure modified from Elgueta *et al.*[120]

Abbreviations – Akt: thymoma viral proto-oncogene 1, Cbl: Casitas B-lineage lymphoma (E3 ubiquitin-protein ligase), JAK3: Janus family kinase 3, JNK: c-Jun N-terminal kinase, NF-κB: nuclear factor κB, p38: mitogen-activated protein kinase p38, PI3K: phosphatidylinositol 3-kinase, STAT5: signal transducer and activator of transcription 5, TRAF: tumor necrosis factor receptor-associated factor.

Part I

C1q/anti-C1q complexes mediate inflammation in PBMCs after T cell activation

Abstract

Objectives

SLE is a clinically heterogeneous autoimmune disease with complex pathogenic mechanisms. Complement C1q has been shown to play a major role in SLE, and anti-C1q are strongly associated with SLE disease activity and severe LN suggesting a pathogenic role for anti-C1q. Whereas C1q alone has anti-inflammatory effects on human monocytes and macrophages, C1q/anti-C1q complexes favor a pro-inflammatory phenotype. This study aims to elucidate the inflammatory effects of anti-C1q on PBMCs.

Methods

Isolated monocytes, isolated T cells, and bulk PBMCs of healthy donors with or without concomitant T cell activation were exposed to C1q or complexes of C1q and SLE patient-derived anti-C1q (C1q/anti-C1q). Functional consequences of C1q/anti-C1q on cells were assessed by determining cytokine secretion, monocyte surface marker expression, T cell activation, and proliferation.

Results

Exposure of isolated T cells to C1q or C1q/anti-C1q did not affect their activation and proliferation. However, unspecific T cell activation in PBMCs in the presence of C1q/anti-C1q resulted in increased TNF, IFNγ, and IL-10 secretion compared to C1q alone. Co-culture and inhibition experiments showed that the inflammatory effect of C1q/anti-C1q on PBMCs was due to a direct CD40–CD154 interaction between activated T cells and C1q/anti-C1q primed monocytes. The CD40-mediated inflammatory reaction of monocytes involves TRAF6 and JAK3-STAT5 signaling.

Conclusion

In conclusion, C1q/anti-C1q have a pro-inflammatory effect on monocytes that depends on T cell activation and CD40–CD154 signaling. This signaling pathway could serve as a therapeutic target for anti-C1q mediated inflammation.

Introduction

SLE is a systemic autoimmune disease with heterogeneous clinical manifestations and complex pathogenic mechanisms.[140] Antibodies against a wide range of autoantigens, formation of immune complexes, and aberrant clearance of apoptotic cells are typical findings in patients with SLE.[105,141,142] The clearance of immune complexes and apoptotic cells involves the complement system, and deficits in molecules of the early classical pathway of complement (i.e., C1q, C1r, C1s, C4, and C2) are strongly associated with SLE.[2,4] Among those deficiencies, homozygous C1q deficiency is the strongest genetic risk factor for disease development.[3,5] However, primary C1q deficiency as a cause of SLE is rare. Most patients suffer from secondary hypocomplementemia, most likely caused by increased complement activation via the classical and lectin pathways associated with the occurrence of anti-C1q.[81–84] These polyclonal, high-affinity autoantibodies recognize neo-epitopes expressed in the CLR of bound C1q.[25,98,143,144] Furthermore, anti-C1q are associated with disease activity, particularly with severe LN. Patients with renal involvement show increasing levels of anti-C1q before a recurring exacerbation and high deposition of anti-C1q in glomeruli.[9,10,91–93] Considering a large number of functions of C1q[38] and the association of anti-C1q with SLE disease manifestations, it is highly likely that anti-C1q have a disease-modifying effect.[97,145] However, the exact means of how anti-C1q contribute to disease activity and LN remain unclear.

In addition to the role as an initiator protein of the classical pathway of complement and pattern recognition molecule, C1q also exerts cellular functions.[146] C1q bound to target patterns (e.g., apoptotic cells, pathogens, cholesterol crystals) facilitates phagocytosis and regulates a wide range of cytokines towards a less inflammatory cytokine secretion profile in human innate immune cells (e.g., monocytes, macrophages, immature DCs).[12,147–151] Regarding anti-C1q, Thanei *et al.* demonstrated that C1q/anti-C1q complexes reverse the phagocytosis enhancing and anti-inflammatory effects of C1q and induce a pro-inflammatory phenotype in HMDMs.[16]

In addition, emerging evidence suggests that C1q exerts an immunosuppressive effect on innate immune cells and T cells.[13,56–59,65] Additionally, T cells have been implicated in SLE as, for example, they make up the majority of cells present in the tubulointerstitial lesions of kidney biopsies of SLE patients.[140,152] The direct interaction of soluble C1q and C1q receptors present on T cells results in less activation, fewer cell divisions, and less cytokine secretion.[13,56,57] Furthermore, Clarke *et al.* reported an indirect route for C1q to modulate T cell activation, proliferation, and

differentiation via macrophages primed with C1q-coated late apoptotic lymphocytes in *in vitro* co-culture experiments.[65]

Taken together, T cells, macrophages, C1q, and anti-C1q play an important role in the course of SLE. Nevertheless, the downstream mechanisms and functional consequences of C1q/anti-C1q complexes are still poorly understood. To better understand anti-C1q in SLE, investigated the immunological effects of C1q/anti-C1q complexes on PBMCs in a setting of activated T cells by studying cytokine secretion, T cell proliferation and activation, and monocyte surface marker expression.

Hypothesis

With our current knowledge about the immunomodulatory capabilities of C1q (e.g., monocytes,[12] macrophages,[12,16] DCs,[12] and T cells[13,56,57,65]) as well as C1q/anti-C1q complexes (e.g., macrophages[16]), we sought to analyze potential immunomodulatory effects in PBMCs and especially T cells in our *in vitro* model of anti-C1q-mediated autoimmunity. We postulated that anti-C1q alter the immunomodulatory effect of C1q in immune cells. Furthermore, we challenged previous data on C1q-mediated effects in T cells and attempted to better understand the immunomodulatory capabilities of bound C1q in the context of C1q/anti-C1q complexes.

Methods

Cell culture

PBMCs – Peripheral blood from healthy donors was collected in ethylenediaminetetraacetic acid tubes at the Blood Transfusion Center of the University Hospital Basel (Basel, Switzerland). PBMCs were isolated by density gradient centrifugation using Lymphoprep (Serumwerk, Bernburg, Germany).

Monocytes – $CD14^+CD16^-$ monocytes were obtained from PBMCs by immunomagnetic negative selection (EasySep™ Human Monocyte Isolation Kit; Stemcell Technologies, Vancouver, Canada), according to the manufacturer's instruction (yielding an average purity of 85–92% viable $CD14^+$ cells in our experiments as determined by flow cytometry). To induce CD40 expression for monocyte/rhabdomyosarcoma (RD) cell co-culture experiments, isolated monocytes were preincubated with 500 U mL^{-1} IFNγ (PeproTech, Cranbury, NJ, USA) in a complete cell culture medium (Roswell Park Memorial Institute [RPMI] medium supplemented with 300 mg mL^{-1} L-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 10% [v/v] fetal calf serum [all from Life Technologies, Carlsbad, CA, USA]) for 18 h.

T cells – Similarly, an immunomagnetic negative selection was used to isolate T cells from PBMCs (EasySep™ Human T Cell Isolation Kit; Stemcell Technologies), according to the manufacturer's instruction (yielding an average purity of 90–95% $CD3^+$ viable cells as determined by flow cytometry).

CD14 depleted PBMCs – To deplete $CD14^+$ cells from PBMCs, an immunomagnetic positive selection kit for $CD14^+$ (CD14 MicroBeads, human; Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturer's instruction leading to an average depletion rate of 89–95% in our experiments (determined by flow cytometry).

RD cells – Human-derived TE671 RD cells from ATCC (American Type Culture Collection; LGC, Wesel, Germany) non-transfected and stably transfected with human CD154 (kind gift from Nicholas Sanderson, Laboratory of Clinical Neuroimmunology, Department of Biomedicine, University of Basel, Basel, Switzerland)[153] were cultured in complete cell culture medium in cell culture bottles (BD Biosciences, Franklin Lakes, NJ, USA) at 37°C and 5% CO_2. Expression of CD154 was confirmed by flow cytometry (Supplementary figure 2a Part I, page 46). Medium renewal and subculturing were performed in a ratio of 1:10 every 3 to 4 days.

Anti-C1q/IgG source

The selection of 20 sera/plasma from SLE patients included in the Swiss Systemic Lupus Erythematosus (SSCS) was based on biomaterial availability, fulfilling at least three of the 11 criteria of the ACR,[154,155] and anti-C1q levels (100-1,000 AU). Of the 20 patients, 16 (80%) were female, and four (20%) were male. The median age at the time of blood sampling was 42 (27.5–40.3) years. Normal human serum (NHS) was obtained from age and sex-matched healthy blood donors from the Blood Transfusion Center of the University Hospital Basel.

To determine cellular mechanisms, anti-C1q positive plasma was obtained from a previously described 20-year-old female SLE patient with active class IV LN at the time of sampling, fulfilling six of the 11 ACR.[156] Anti-C1q levels in this individual were quantified by a previously described anti-C1q ELISA (1,000 AU, cut-off value 50 AU)[98,144,157] and confirmed by a commercially available anti-C1q ELISA kit (Bühlmann, Schönenbuch, Switzerland) used in our clinical routine laboratory (2,599 U mL^{-1}, cut-off 15 U mL^{-1}). For comparison, anti-C1q negative (< 5 AU) plasma of an age-matched healthy female donor was included as a negative control. The local Ethics Committee approved the sampling and use of blood samples included in this study (EKZ No. 110/04; 130/05).

In vitro model of anti-C1q autoimmunity

The *in vitro* model of anti-C1q autoimmunity was used as described before.[16,22,158] Briefly, flat-bottom 96-well plates (Eppendorf, Hamburg, Germany) were coated with 70 µL of 5 µg mL^{-1} purified human C1q (Complement Technology, Tyler, Tx, USA) in coating buffer (0.4 M sodium carbonate buffer, pH 9.6) overnight at 4°C. The plates were washed twice with 140 µL phosphate-buffered saline (PBS, Life Technologies) before adding anti-C1q positive (SLE) or anti-C1q negative (NHS) sera. Each serum sample was centrifuged at 14,000 *g* at 4°C for 30 min and diluted at 1:100 in PBS 1 M NaCl (Sigma-Aldrich, St. Louis, MO, USA) before incubation on a shaker (500 rounds per minute [rpm]) at room temperature for 1 h. Again, plates were washed four times with 140 µL PBS. Next, PBMCs (200,000 $well^{-1}$), T cells (200,000 $well^{-1}$), or monocytes/T cells (20,000 monocytes and 100,000 T cells $well^{-1}$) were added and activated with 5 µL mL^{-1} soluble tetrameric anti-CD3/anti-CD28 complex (ImmunoCult™ Human CD3/CD28 T cell activator; Stemcell Technologies) in a final volume of 200 µL for 24 h.

T cell proliferation

To assess T cell proliferation, T cells were labeled with 5 µM carboxyfluorescein succinimidyl ester (CFSE, Biolegend, San Diego, CA, USA) at 37°C for 10 min and quenched five times with a complete cell culture medium. T cell proliferation was analyzed by flow cytometry after 96 h. Percent dividing cells and the proliferation index were calculated by FlowJo 10.7.1 (BD Biosciences) and used to describe T cell proliferation.[159]

Cytokine quantification

Cell culture supernatants were collected after the indicated experiment period, centrifuged (1,000 *g*, 4°C, 10 min) to remove cell debris and stored at -80°C until further quantification. Commercially available ELISA kits for TNF (BD Biosciences), IL-10 (Biolegend), and IFNγ (Immunotools, Friesoythe, Germany) were used to measure cytokine concentrations according to the manufacturer's instructions.

Anti-C1q quantification

Anti-C1q ELISA was performed as previously published.[81,98,102,157] In brief, ELISA plates were coated with 5 µg mL^{-1} purified human C1q. Blood samples were diluted at 1:50 in high-salt buffer (PBS 1 M NaCl with 0.05% Tween 20 [Sigma-Aldrich]) and added to the C1q coated wells for 1 h at 37°C. To detect bound IgG, alkaline phosphatase (AP)-conjugated rabbit anti-human IgG (Promega, Madison, WI, USA) was used. Absorbance at 405 nm was read using a microplate ELISA reader (BioTek Instruments, Winooski, Vt, USA). Anti-C1q levels were calculated using a reference SLE sample (set as 1,000 arbitrary units [AU]).

Quantification of C3 deposition

The polystyrene cell culture plates were coated with 70 µL of 5 µg mL^{-1} purified human C1q in coating buffer overnight at 4°C. The plates were washed three times with 140 µL PBS 0.05% (v/v) Tween-20 after each incubation step. To block unoccupied binding sites, 140 µL PBS 1% (m/v) bovine serum albumin (BSA, Sigma-Aldrich) were added for 1 h at room temperature. Prior to dilution, serum samples were centrifuged at 14,000 *g* at 4°C for 30 min. Serum samples were diluted at 1:100 in HEPES buffered saline (HBS, supplemented with 2.5 mM HEPES [AppliChem GmbH, Darmstadt, Germany], 500 µM $MgCl_2$ [Sigma-Aldrich], and 150 µM $CaCl_2$ [Sigma-Aldrich]; pH 7.4), HEPES 1 M NaCl, PBS (lacking magnesium and calcium; pH 7.4), and PBS 1 M NaCl. Next, 70 µL of the diluted samples were added to the plates for 1 h at 37°C. To measure

C3 deposition, a polyclonal goat anti-human C3 antibody (Quidel, San Diego, CA, USA) and a mouse anti-goat horseradish peroxidase (HRP)-conjugated antibody (Sigma-Aldrich) were added for 1 h each at room temperature in a dilution of 1:10,000 and 1:40,000, respectively. The enzyme activity was assessed by the addition of 3,3', 5,5' tetramethylbenzidine (TMB, BD Biosciences). After 20 min the enzymatic reaction was stopped with 70 µL of 4 M sulfuric acid and the absorbance at 450 nm was read using a microplate ELISA reader. Finally, the blank values were subtracted for each sample and buffer.

Flow cytometry

After 24 h of stimulation on different coatings described above, cells were collected for analysis by flow cytometry. To exclude dead cells, either 4',6-diamidino-2-phenylindole (DAPI, 3 µM; Biolegend) or Fixable Viability Dye eFluor 780 (Thermo Fisher Scientific, Waltham, MA, USA) was used according to the manufacturer's instructions. To avoid unspecific binding of IgG, cells were incubated with 2 mg mL^{-1} human IgG (Blood Transfusion Service SRC, Bern, Switzerland) per 1,000,000 cells in fluorescence-activated cell sorting (FACS) buffer (PBS supplemented with 1% [m/v] BSA and 1 mM sodium azide [Sigma-Aldrich]) at 4°C for 30 min. Additionally, appropriate biological and/or isotype controls were applied to ensure the specificity of the antibodies. Staining for surface marker expression was performed for 30 min at 4°C and included the following antibodies: mouse anti-human CD25 allophycocyanin (APC) and CD69 phycoerythrin (PE) (both from Immunotools) (antibody panel 1), or CD11c APC (Biolegend), CD40 fluorescein isothiocyanate (FITC), CD80 FITC, and CD86 FITC (all from BD) (antibody panel 2). After washing cells twice with FACS buffer, at least 20,000 events in the viable gate were acquired on a BD LSRFortessa (BD Biosciences) and analyzed using FlowJo 10.7.1 to calculate mean fluorescence intensity (MFI).

For detection of intracellular TNF, IFNγ, and IL-10 in PBMCs after 24 h, brefeldin A (3 µg mL^{-1}; eBioscience, San Diego, CA, USA) was added for the final 4 h of cell culture. Following extracellular staining with CD4 Brilliant Violet™ (BV) 510, CD8 BV711, CD14 PE/Cyanine (Cy)7, CD19 BV421, and CD56 Alexa Fluor 488 (all from Biolegend) (antibody panel 3) for 30 min at 4°C, cells were fixed and permeabilized using Intracellular Fixation & Permeabilization Buffer Set (eBioscience) according to the manufacturer's instruction. Next, cells were incubated for 45 min at room temperature with mouse anti-human TNF PE, IFNγ BV605, and rat anti-human IL-10 APC (all from Biolegend). A minimum of 100,000 events in the viable gate were acquired on a Cytek Aurora (Cytek Biosciences, Fremont, CA, USA).

Inhibition of intracellular TRAF6 and JAK3-STAT5 CD40 signaling

TRAF6 specific inhibitor 6877002 (IC_{50} 15.9 μM)[160], NFκB specific inhibitor JSH-23 (IC_{50} 7.1 μM)[161], and JAK3 specific inhibitor PF-06651600 (IC_{50} 33.1 nM)[131] were dissolved in dimethyl sulfoxide (DMSO) and deionized water (all from Sigma-Aldrich), respectively, and sterile filtered using a 0.22 μm mixed cellulose ester membrane filter (Merck, Burlington, VT, USA). Final DMSO concentrations did not exceed 0.3% (v/v) in cell culture experiments.

Statistical analysis

The non-parametric statistical analyses between two groups were performed using the Wilcoxon signed-rank and Mann-Whitney *U*-test for paired and unpaired data; differences between multiple groups were determined using the Friedman test following the Tukey's multiple comparison test. Correlations were calculated using Spearman's rho. Statistical significance was considered with $*P \leq 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Analyses were conducted with GraphPad Prism 9.1.2 (GraphPad Software, San Diego, CA, USA).

Results

Anti-C1q bound to C1q increase cytokine secretion in PBMCs after T cell activation

We first analyzed the effect of C1q/anti-C1q complexes on PMBCs in a non-septic chronic inflammatory setting. For this purpose, PBMCs were cultured for 24 h on bound C1q preincubated with anti-C1q negative sera (C1q/NHS) from healthy donors and bound C1q preincubated with anti-C1q positive sera from SLE patients (C1q/anti-C1q).

To induce an inflammatory milieu, PBMCs were simultaneously activated by a dose of 5 μL mL^{-1} soluble human CD3/CD28 T cell activator. In contrast to the commonly used surface or bead bound anti-CD3/CD28 T cell activators, there was no interference between C1q and the soluble tetrameric complex structure used in our *in vitro* model (Figure 4a and b). Additionally, the presence of human serum in PBS 1 M NaCl (deficient in calcium and magnesium) did not lead to the activation of the complement cascade and potential attachment of complement onto the plate as shown by the lack of C3 deposition (Figure 4c).

PBMCs significantly upregulated TNF ($P < 0.0001$), IFNγ ($P < 0.0001$), and IL-10 ($P = 0.0003$) secretion in the presence of C1q/anti-C1q complexes obtained from 20 SLE patients compared to C1q/NHS (Figure 5, left). Additionally, levels of anti-C1q in SLE patients were found to correlate with TNF ($r = 0.6592$), IFNγ ($r = 0.6349$), and IL-10 ($r = 0.5226$) concentrations (Figure 5, right). Intra-patient comparison of anti-C1q negative (< 50 AU) and anti-C1q positive (≥ 50 AU) sera from separate time points revealed equivalent increases in TNF, IFNγ, and IL-10 as shown in C1q/NHS and C1q/anti-C1q (Figure 6). Based on this observation, further experiments elucidating involved cell types and cellular mechanisms were standardized to the use of anti-C1q positive plasma from a previously published patient with 1,000 AU anti-C1q.[156]

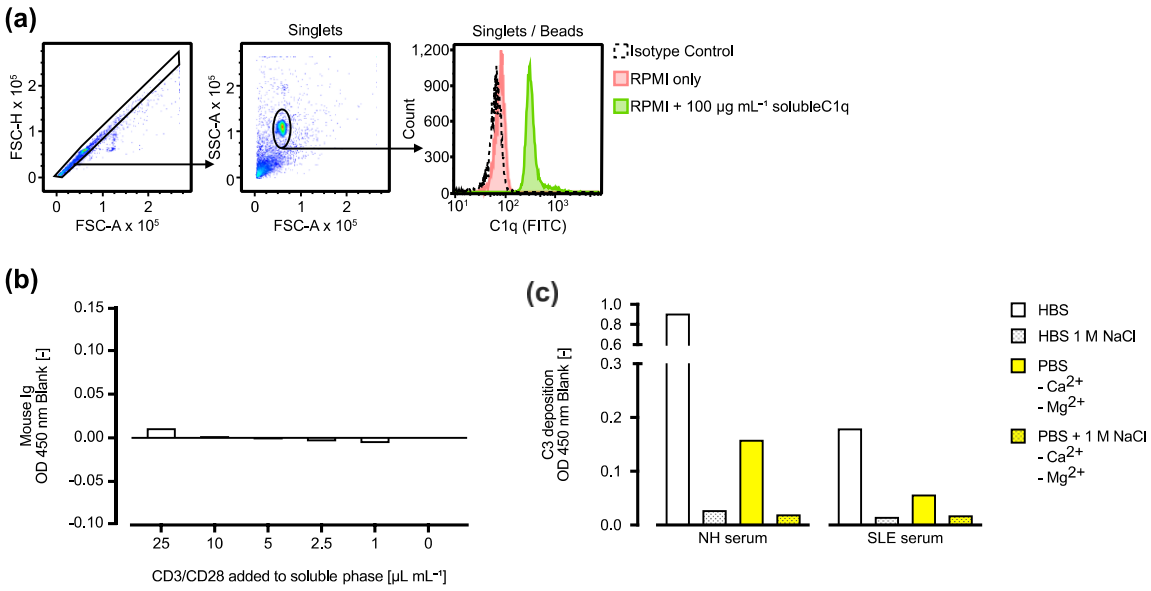


Figure 4 | C1q interacts with bound anti-CD3/CD28 T cell activating antibodies, whereas soluble tetrameric anti-CD3/CD28 antibody complexes do not. Additionally, complement activation was not observed with PBS 1 M NaCl buffer.

(a) Dynabeads coupled to anti-CD3/CD28 antibodies (Life Technologies) were incubated in complete cell culture medium supplemented with or without 100 µg mL^{-1} soluble C1q at 37°C. After 96 h, binding of C1q to Dynabeads was assessed using flow cytometry. **(b)** Similarly, binding of soluble tetrameric anti-CD3/CD28 complexes (Stemcell Technologies) to bound C1q was analyzed by an anti-mouse Ig ELISA. **(c)** Complement C3 deposition was measured by ELISA to determine complement activation in normal human (NH) and SLE patient (SLE) samples in the presence of bound C1q. Samples were diluted 1:100 in HEPES buffered saline supplemented with calcium and magnesium (HBS, pH 7.4), HBS 1 M NaCl, PBS lacking calcium and magnesium (pH 7.4), or PBS 1 M NaCl.

Because the effect of the C1q/anti-C1q complexes might be solely attributed to the interaction of IgG and Fc receptors on monocytes, we exposed PBMCs to coated monomeric IgG (5 µg mL^{-1}) of anti-C1q negative and positive samples, respectively. Interestingly, the presence of purified anti-C1q positive IgG alone did not increase TNF secretion compared to anti-C1q negative IgG or C1q alone, whereas purified anti-C1q positive IgG complexed with C1q elevated TNF secretion (Figure 7a and b). As a control for the specificity of the C1q/anti-C1q complexes, an alternative immune complex consisting of human serum albumin (HSA)/anti-HSA was also tested. Again, TNF secretion in PBMCs did not significantly differ between HSA/anti-HSA complexes and bound HSA alone (Figure 7c).

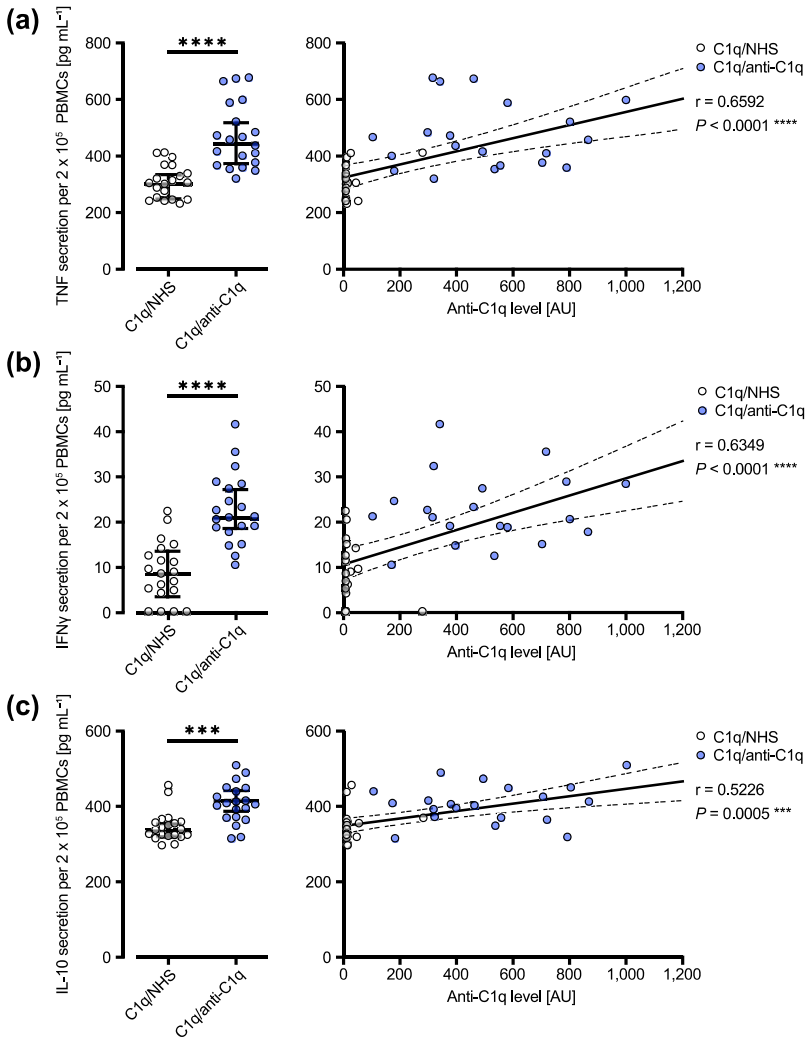


Figure 5 | Increase in TNF, IFNγ, and IL-10 secretion in PBMCs after T cell activation and correlation with anti-C1q levels

PBMCs activated by tetrameric anti-CD3/CD28 complexes were cultured on C1q preincubated with the serum of 20 SLE patients (C1q/anti-C1q) or with the serum of 20 healthy donors (C1q/NHS) for 24 h. Cell culture supernatants were analyzed for **(a)** TNF, **(b)** IFNγ, and **(c)** IL-10 secretion by ELISA. Data points represent the median cytokine concentration obtained from PBMCs of four unrelated healthy donors from independent experiments exposed to one single serum sample. (Left) Horizontal lines with error bars show median with interquartile range (IQR). The Mann-Whitney *U*-test, ***$P < 0.001$, ****$P < 0.0001$. (Right) Solid line represents linear regression with dotted lines indicating the 95% confidence bands. Spearman's rank correlation of cytokine secretion and anti-C1q levels, ***$P < 0.001$, ****$P < 0.0001$.

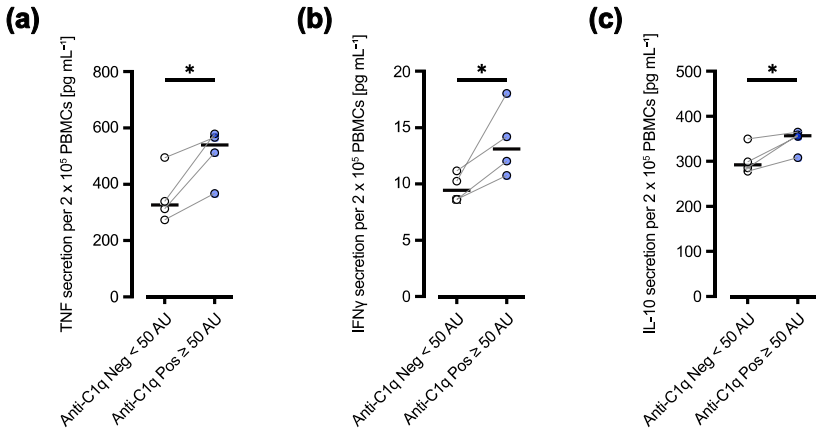


Figure 6 | Increase in TNF, IFNγ, and IL-10 in intra-patient comparison of anti-C1q negative and positive samples from different time points

PBMCs activated by tetrameric anti-CD3/CD28 complexes were cultured on C1q preincubated with anti-C1q negative and positive sera each from the same patient ($n = 4$) but obtained at different time points at patient's history (i.e., intra-patient comparison) for 24 h. Cell culture supernatants were analyzed for **(a)** TNF, **(b)** IFNγ, and **(c)** IL-10 secretion by ELISA. Data points represent the median cytokine concentration obtained from PBMCs of four unrelated healthy donors from independent experiments exposed to one single serum sample. Horizontal lines represent median cytokine secretion. Connecting lines link serum samples of the same patient from different time points. Paired *t*-test, $*P \leq 0.05$.

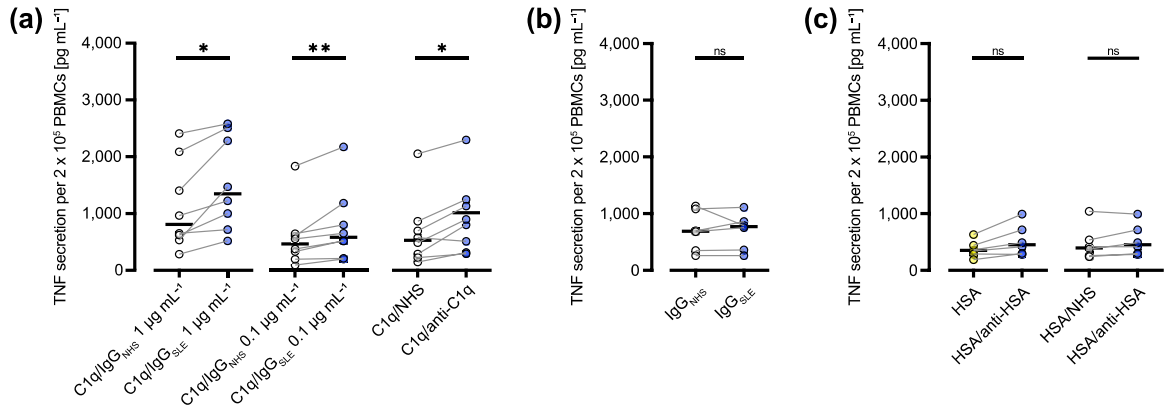


Figure 7 | Increase in TNF secretion in PBMCs after T cell activation specific to C1q/anti-C1q complexes

PBMCs activated by tetrameric anti-CD3/CD28 complexes were cultured on **(a)** NHS or SLE patient-derived purified IgG complexed with C1q, or **(b)** coated NHS or SLE patient-derived purified IgG (5 µg mL^{-1}) alone, or **(c)** HSA preincubated with anti-HSA positive NHS (HSA/anti-HSA), HSA preincubated with anti-HSA negative NHS (HSA/NHS), respectively. After 24 h, cell culture supernatants were analyzed for cytokine secretion by ELISA. Median cytokine concentrations of TNF are shown as horizontal lines. Data points represent independent experiments analyzing PBMCs from **(a)** eight and **(b and c)** six different healthy donors. Connecting lines link data points of a single donor used to obtain PBMCs. Wilcoxon matched-rank test, $*P \leq 0.05$, $**P < 0.01$, ns: not significant.

Bound C1q does not affect T cells directly

Previous studies suggest a direct anti-proliferative and anti-inflammatory effect of soluble C1q on T cells.[33,35,38,41] To investigate the potential direct effect of bound C1q and C1q/anti-C1q complexes, respectively, on T cell activation and proliferation, we incubated isolated CD3$^+$ T cells with bound HSA (1 µg well^{-1}), bound C1q (1 µg well^{-1}), C1q/anti-C1q complexes and uncoated wells in the presence of soluble C1q (100 µg mL^{-1}). Bound C1q did not significantly modulate the secretion of TNF and IL-10 as well as the expression of activation markers CD25 and CD69 after 24 h of T cell activation when compared to HSA (Figure 8a-d). However, TNF secretion was significantly decreased in the presence of soluble C1q compared to HSA after 24 h (Figure 8a). No significant difference in T cell proliferation was observed between bound HSA, bound C1q, bound C1q/anti-C1q, and soluble C1q exposure after 96 h (Figure 8e and f, gating strategy Supplementary figure 1a Part I, page 45).

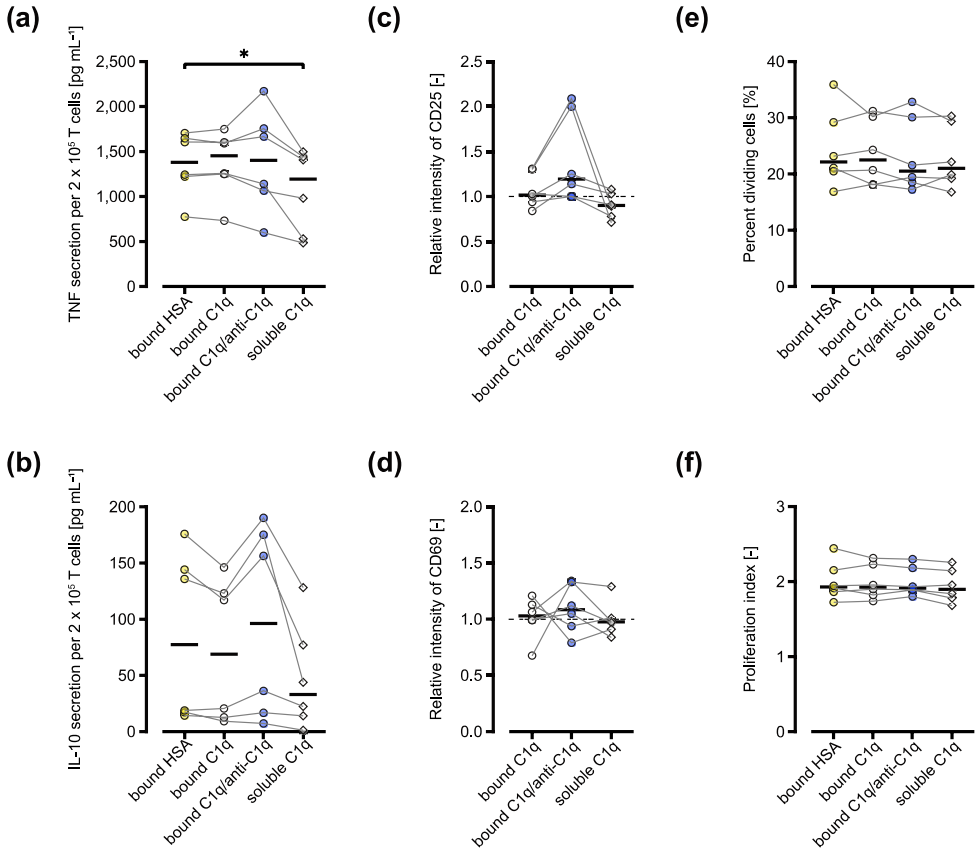


Figure 8 | T cell proliferation, activation, and IL-10 secretion in activated T cells not affected by bound C1q, bound C1q/anti-C1q, and soluble C1q, respectively, whereas TNF secretion decreased in the presence of soluble C1q

T cells were isolated from PBMCs of healthy donors and cultured on bound HSA, bound C1q, bound C1q preincubated with anti-C1q positive SLE serum (bound C1q/anti-C1q), or together with soluble C1q without coating. T cells were activated by tetrameric anti-CD3/CD28. Cytokines **(a)** TNF and **(b)** IL-10 as well as activation markers **(c)** CD25 and **(d)** CD69 were analyzed after 24 h by ELISA and flow cytometry, respectively. For proliferation assessment, cells were stained with CFSE prior to the experiment and **(e)** percent dividing cells and **(f)** proliferation index were analyzed by flow cytometry after 96 h. Data points represent six different healthy donors used to obtain PBMCs analyzed in independent experiments with connecting lines linking data points of a single individual. Median values are shown as solid horizontal lines. The Friedman test with Dunn's posttest correction (all vs bound HSA), $*P \leq 0.05$. **(c and d)** Relative intensity is calculated by normalizing MFI of bound C1q, bound C1q/anti-C1q, and soluble C1q to bound HSA. The horizontal dashed line marks the relative change in intensity of 1.0 (Supplementary figure 1a Part I [page 45] depicts the gating strategy).

The presence of CD14$^+$ cells is essential for increased TNF secretion in the presence of C1q/anti-C1q complexes

We next performed an intracellular cytokine staining after 24 h of anti-CD3/CD28 stimulation to evaluate the source of the observed cytokines. Categorization of CD4, CD8, CD14, CD19, CD56,

and other cell types revealed that the main TNF and IL-10 producing cells are $CD14^+$ monocytes (TNF: $P < 0.001$; IL-10: $P = 0.017$) when exposed to C1q/anti-C1q compared to C1q/NHS (Figure 9). With these results and the fact that a previous study on HMDMs found a pro-inflammatory cytokine secretion profile in the presence of C1q/anti-C1q complexes,[32] we next investigated whether the interaction between monocytes and activated T cells accounts for the observed increase in TNF, IFNγ, and IL-10. For this, we performed autologous co-culture experiments of isolated $CD3^+$ T cells and $CD14^+CD16^-$ monocytes with concomitant anti-CD3/CD28 stimulation of T cells for 24 h. Unlike IL-10 secretion, the increase in TNF and IFNγ in the co-culture setting after exposure to C1q/anti-C1q complexes was identical to the observation made in PBMCs, suggesting that monocytes are essential for the secretion of these cytokines (Figure 10a and b, left and middle panel). In contrast, IL-10 secretion seems to require the interaction of further immune cells (Figure 10c, left and middle panel). In line with this finding, depletion of $CD14^+$ cells (89–95% efficiency) abolished the pro-inflammatory effect for IFNγ ($P = 0.625$) and greatly reduced the increase observed in TNF ($P = 0.031$) and IL-10 secretion ($P = 0.031$) (Figure 10, right panel), suggesting that the remaining $CD14^+$ monocytes are sufficient for a moderate but still significant increase in TNF and IL-10.

Taken together, the interaction between activated T cells and monocytes is responsible for increased TNF and IFNγ levels in the presence of C1q/anti-C1q complexes, whereas increased IL-10 levels require further signals from cells present in PBMCs but missing in the co-culture of monocytes and T cells.

Direct cell–cell contact between monocytes and T cells mediates TNF secretion through CD40–CD154 binding

To evaluate whether the increased TNF concentration in co-cultured monocytes and T cells requires direct cell–cell contact or is mediated via soluble factors, we expanded the co-culture to a transwell experiment. For this, monocytes were exposed to C1q/NHS and C1q/anti-C1q coatings, whereas culturing and activation with anti-CD3/CD28 of T cells for 24 h occurred in inserts separated from the monocytes and coatings. Differences in TNF secretion between C1q/NHS and C1q/anti-C1q settings disappeared after the separation of monocytes and T cells, indicating that cell–cell contact is required (Figure 11a).

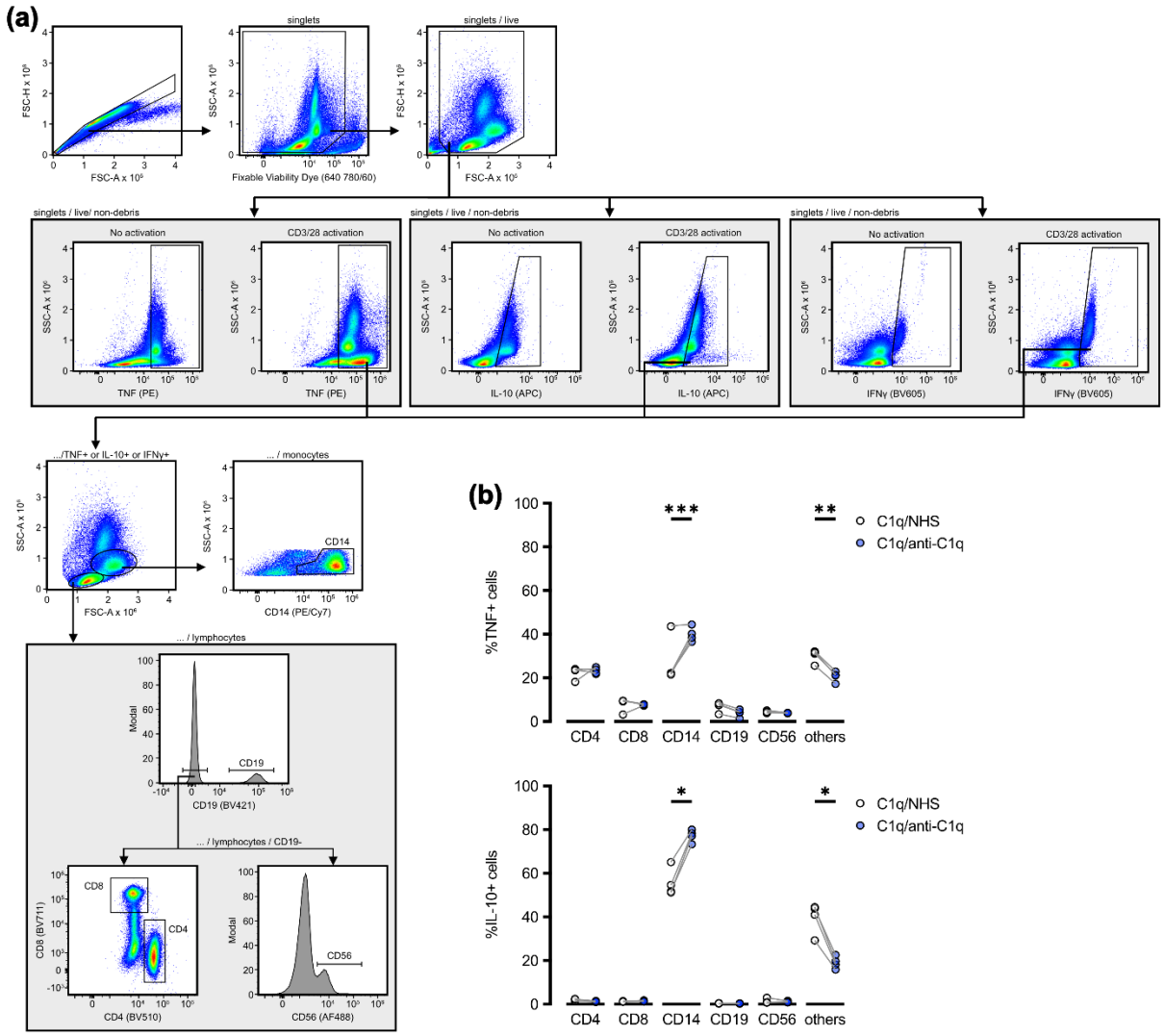


Figure 9 | Detection of intracellular cytokines in PBMCs following T cell activation

PBMCs activated by tetrameric anti-CD3/CD28 complexes were cultured on C1q preincubated with anti-C1q positive SLE patient (C1q/anti-C1q) or anti-C1q negative healthy donor (C1q/NHS) derived serum for 24 h. Brefeldin A (3 µg mL^{-1}) was added for the final 4 h before cells were analyzed for intracellular TNF, IL-10, and IFNγ by flow cytometry. **(a)** Gating strategy used to obtain percentage of cells positive for TNF, IL-10 or IFNγ. Flow cytometry dot plots and histograms show one donor representative for four healthy donors. **(b)** Data are reported as percentage of TNF or IL-10 positive cells of four unrelated healthy donors used to obtain PBMCs. Two-way analysis of variance (ANOVA) test, $*P \leq 0.05$, $**P < 0.01$, $***P < 0.001$.

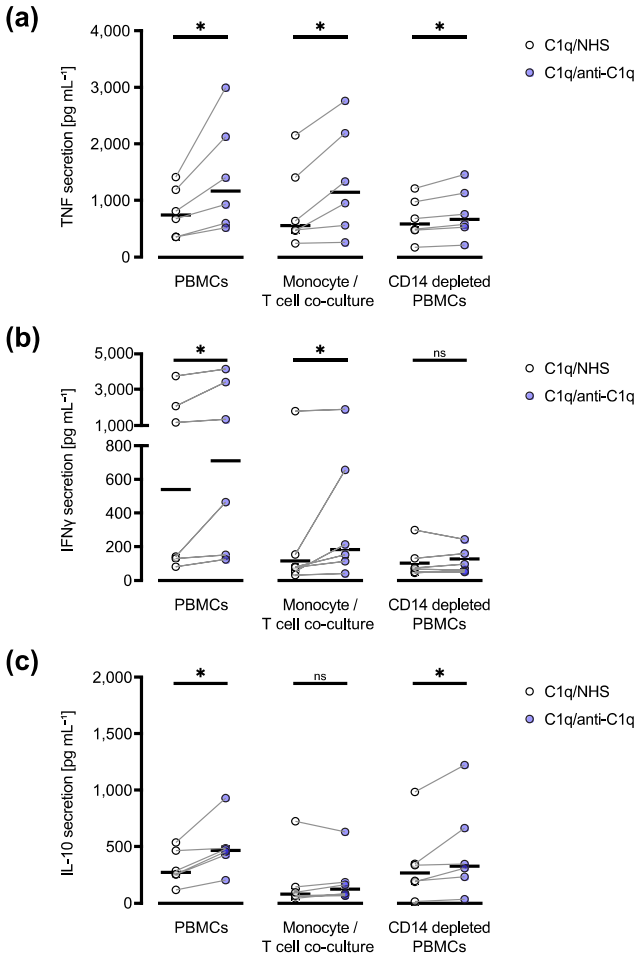


Figure 10 | The presence of CD14⁺ cells is essential for the increased cytokine secretion in the presence of C1q/anti-C1q complexes

PBMCs, monocytes and T cells (co-culture 1:5 ratio), and CD14 depleted PBMCs (89-95% efficacy) were cultured on C1q preincubated with anti-C1q negative NHS (C1q/NHS) or anti-C1q positive SLE serum (C1q/anti-C1q), and activated by tetrameric anti-CD3/CD28 complexes for 24 h. Cell culture supernatants were analyzed by ELISA for (a) TNF, (b) IFNγ, and (c) IL-10 secretion. Median cytokine concentrations are shown as horizontal lines and data points represent independent experiments analyzing six different healthy donors used to obtain PBMCs with connecting lines linking data points of a single individual. The Wilcoxon matched-rank test, *P ≤ 0.05, ns: not significant.

Next, we aimed to assess surface markers on monocytes present in the immune synapse of monocytes and T cells. Therefore, we analyzed CD40, CD80, and CD86 expression on $CD11c^+$ cells after T cell activation in PBMCs. After 24 h, CD80 and CD86 levels did not differ in $CD11c^+$ cells between C1q/NHS and C1q/anti-C1q coatings (CD80: $P = 0.562$, CD86: $P = 0.688$). However, CD40 was slightly downregulated in the presence of C1q/anti-C1q complexes

compared to C1q/NHS (CD40: $P = 0.031$) (Figure 11b, gating strategy Supplementary figure 1b Part I, page 45).

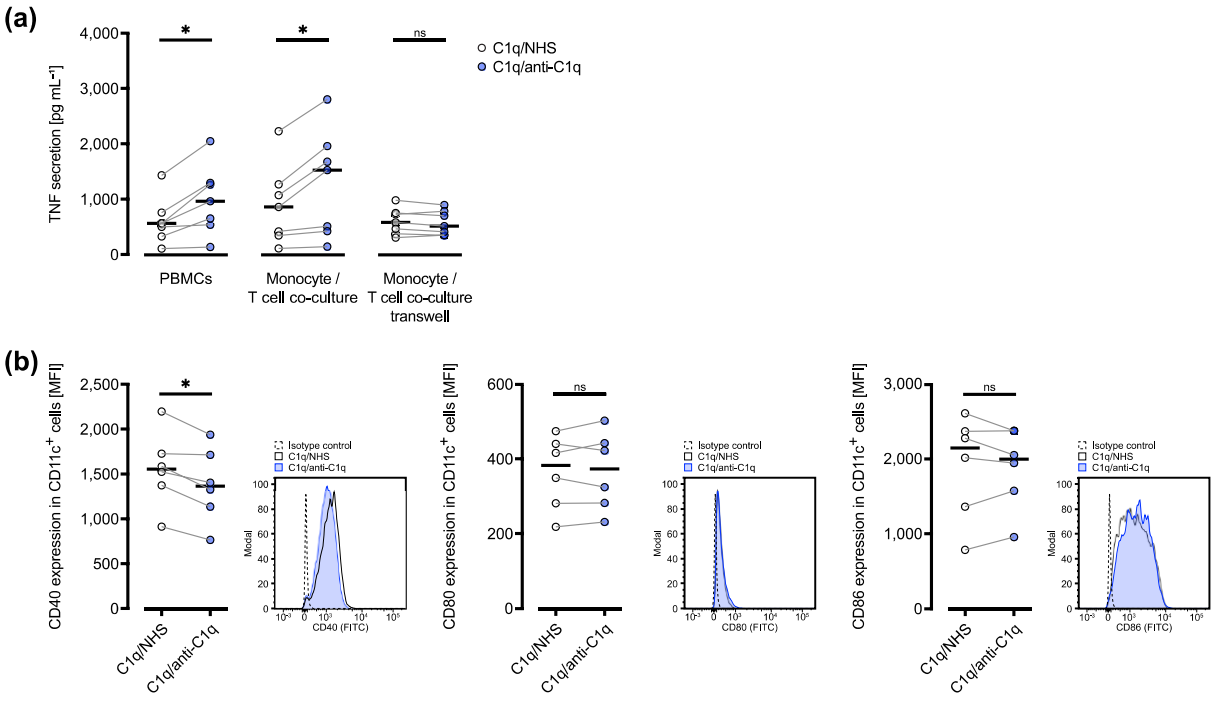


Figure 11 | Increase in TNF secretion after exposure to C1q/anti-C1q complexes requires cell-cell contact between monocytes and T cells and involves CD40 downregulation in CD11⁺ cells

(a) PBMCs or monocytes and T cells (1:5 ratio) co-cultured either together or separated by 0.4 µm pore polyester membrane inserts (monocytes in the receiver plate, T cells in the permeable support system) were exposed to bound C1q, which was preincubated with anti-C1q negative NHS (C1q/NHS) or anti-C1q positive SLE serum (C1q/anti-C1q). Cells were activated with tetrameric anti-CD3/CD28 complexes for 24 h. Cell culture supernatants were analyzed for TNF secretion by ELISA. Median cytokine concentrations are shown as horizontal lines and data points represent seven different healthy donors analyzed in independent experiments. **(b)** Analyses of CD40, CD80, and CD86 in CD11c⁺ cells were performed by flow cytometry after 24 h of cell culture. Median MFIs are shown as horizontal lines and data points represent six different healthy donors analyzed in independent experiments. Connecting lines link data points of a single donor used to obtain cells. The Wilcoxon matched-rank test, $*P \leq 0.05$, ns: not significant. Flow cytometry histograms show one donor representative for six healthy donors (Supplementary figure 1b Part I [page 45] depicts the gating strategy).

Previous studies showed the importance of the CD40–CD154 interaction in T cell-mediated immune responses and activation of macrophages.[42–45] Therefore, we explored this interaction in our setting. For this purpose, PBMCs were cultured and T cells activated as described above in the presence of either a mouse anti-CD154 blocking antibody (5 µg mL^{-1}; clone 24-31, Life Technologies) or an isotype control (5 µg mL^{-1}; clone P3.6.2.8.1, Life Technologies). The addition of the CD154 blocking antibody resulted in a decrease in TNF secretion compared to the isotype

control antibody and the disappearance of a significant difference in TNF secretion between C1q/NHS and C1q/anti-C1q priming ($P = 0.094$) (Figure 12a, right panel).

Considering that the inhibition of CD154 normalized TNF secretion caused by the presence of C1q/anti-C1q, we next assessed if the CD40 signaling in monocytes is sufficient for the observed differences in TNF secretion. For this purpose, isolated monocytes were cultured on C1q/NHS or C1q/anti-C1q. Since unstimulated monocytes express only very low levels of CD40 (Supplementary figure 2b Part I, page 46) compared to PBMCs with activated T cells (Figure 11b), additional priming with 500 U mL^{-1} IFNγ for 18 h was necessary to achieve comparable CD40 levels in isolated monocytes.[46] Afterwards, CD154 expressing RD cells were added in a 2 to 1 ratio (monocytes/RD cells) for 24 h to activate monocytes and mimic activated CD154 expressing T cells. CD154 stimulated monocytes cultured on C1q/anti-C1q increased TNF secretion compared to monocytes on C1q/NHS ($P = 0.008$), confirming the CD40–CD154 interaction to be an important signal for TNF secretion after exposure of monocytes to C1q/anti-C1q complexes (Figure 12b). In a controlled setting with non-transfected RD cells, monocytes did not secrete detectable levels of TNF (Supplementary figure 2c Part I, page 46).

In summary, our data demonstrate that despite the slight downregulation of CD40, the CD40–CD154 signaling axis is sufficient for the upregulation of TNF secretion in monocytes that encountered C1q/anti-C1q complexes.

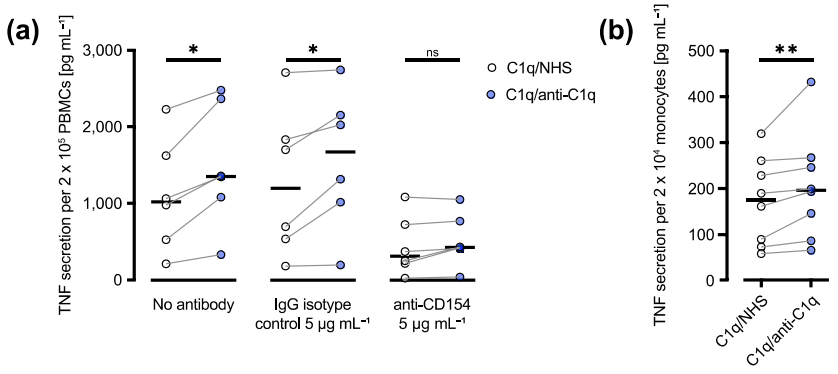


Figure 12 | CD40 signaling to monocytes essential for increased TNF secretion after exposure to C1q/anti-C1q complexes

(a) PBMCs from healthy donors were cultured in the presence of C1q, which was preincubated with anti-C1q negative NHS (C1q/NHS) or anti-C1q positive SLE serum (C1q/SLE) for 24 h. The addition of a blocking mouse anti-CD154 IgG antibody (5.0 µg mL^{-1}) showed a decrease in TNF secretion compared to the isotype control (5 µg mL^{-1}). **(b)** CD40–CD154 signaling to monocytes was confirmed by co-culturing IFNγ primed (500 U mL^{-1}, 18 h) monocytes to CD154 expressing RD cells in the presence (C1q/anti-C1q) or absence (C1q/NHS) of C1q/anti-C1q complexes for 24 h. Cell culture supernatants were analyzed for TNF secretion by ELISA. Median cytokine concentrations of TNF are shown as horizontal lines. Data points represent **(a)** six and **(b)** eight different healthy donors analyzed in independent experiments. Connecting lines link data points of a single donor used to obtain PBMCs. The Wilcoxon matched-signed test, $*P \leq 0.05$, ns: not significant.

JAK3-STAT5 and TRAF6 are partially redundant intracellular CD40 signaling pathways responsible for TNF secretion in anti-C1q primed monocytes

Intracellular CD40 signaling is divided into TRAF dependent and independent signaling, including the JAK3-STAT5 pathway.[47] Both can participate in the induction of TNF in monocytes.[48]

To assess intracellular pathways in our *in vitro* autoimmune model, we co-cultured IFNγ-primed isolated monocytes and CD154 expressing RD cells as described before. However, before the addition of CD154 expressing RD cells, monocytes were treated with either the JAK3 inhibitor PF-06651600 (0–10 µM), TRAF6 inhibitor 6877002 (0–20 µM), or NFκB inhibitor JSH-23 (0–30 µM) for 4 h. Additionally, combinations of PF-06651600 (0–10 µM) plus TRAF 6 inhibitor 6877002 (20 µM) or JSH-23 (30 µM), or a combination of PF-06651600 (0–10 µM) plus TRAF 6 inhibitor 6877002 (20 µM) and JSH-23 (30 µM) were applied to detect potential cumulative effects.

All three inhibitors blocked TNF secretion dose-dependently, achieving an approximately 42–54% reduction at most (Figure 13a-c). Notably, the combination of the JAK3 inhibitor PF-06651600 and the TRAF 6 inhibitor 6877002 further decreased TNF secretion to 27% of the

baseline (Figure 13d), whereas the addition of the NFκB inhibitor JSH-23 to PF-06651600 did not further decrease TNF secretion (Figure 13e and f). Notably, all three inhibitors did not affect cell viability (Supplementary figure 3 Part I, page 47).

These data demonstrate that TNF secretion in CD40-activated IFNγ-primed monocytes is mediated by multiple and partially additive intracellular signaling pathways, with TRAF6 and JAK3-STAT5 signaling being the least redundant.

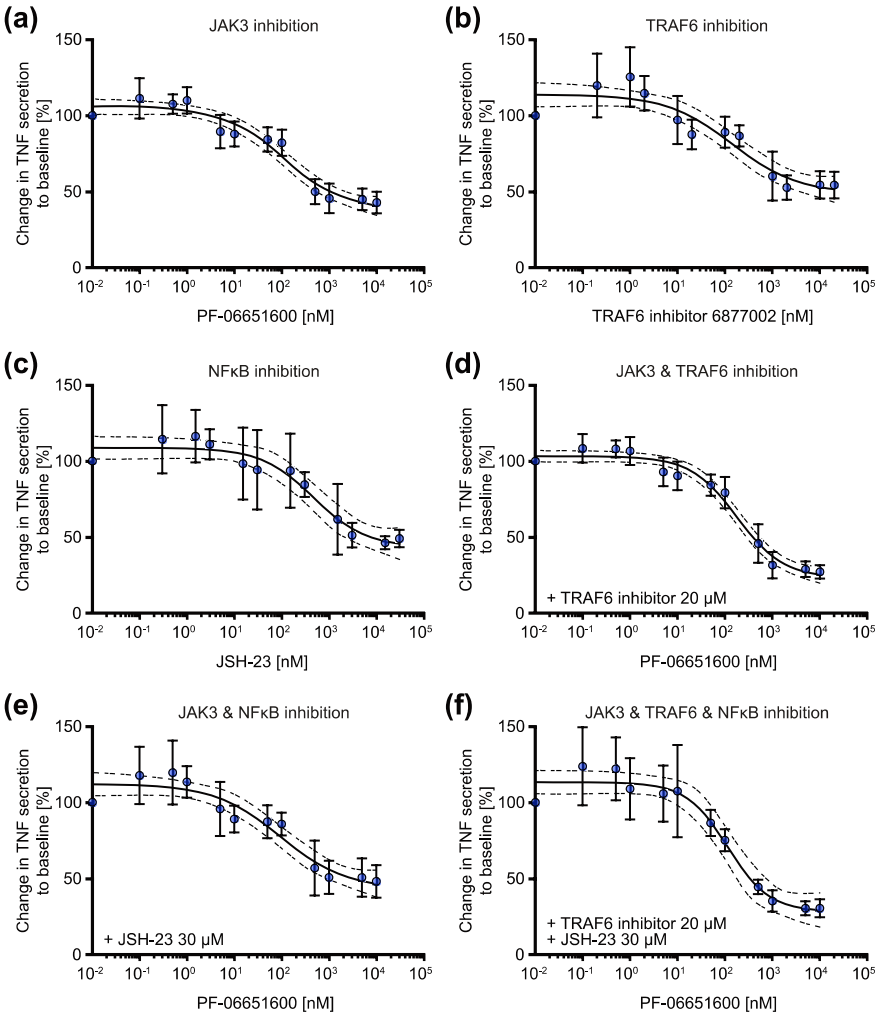


Figure 13 | TNF secretion occurs via partially redundant intracellular CD40 signaling pathways JAK3-STAT5 and TRAF6

Monocytes were isolated from PBMCs of healthy donors and cultured in the presence of C1q/anti-C1q. Cells were preincubated with 500 U mL^{-1} IFNγ for 18 h. Prior to the addition of CD154 expressing RD cells, monocytes were treated with **(a)** PF-06651600 0-10 µM (JAK3 inhibitor), **(b)** TRAF6 inhibitor 6877002 0-20 µM, **(c)** JSH-23 0-30 µM (NFκB inhibitor), **(d)** PF-06651600 0-10 µM plus TRAF6 inhibitor 6877002 20 µM, **(e)** PF-06651600 0-10 µM plus JSH 30 µM, and **(f)** PF-06651600 0-10 µM plus TRAF6 inhibitor 6877002 20 µM and JSH-23 30 µM for 4 h. Cell culture supernatants were analyzed for TNF secretion by ELISA after 24 h of monocyte/RD cell co-culture. Data points represent mean inhibition of TNF secretion, normalized to the secretion without addition of any inhibitor, of **(a-e)** six and **(f)** four different healthy donors. Error bars show standard deviations, solid lines show a four parametric nonlinear regression, and dashed lines the 95% confidence bands.

Discussion

Anti-C1q are considered to play a pathogenic role in the development and maintenance of SLE.[23,24] Anti-C1q correlate with disease activity and can be found in C1q/anti-C1q complexes in the glomeruli of SLE patients with severe LN.[17,21,49] However, the pathogenic impact of anti-C1q and C1q/anti-C1q complexes in particular, on the disease is not well defined. Accumulating evidence indicates that kidney damage is not solely caused by (auto-)antibodies but involves immune cells, including myeloid, T, NK, and B cells[50] present in the glomeruli of LN biopsies as mediators of direct tissue damage.[51–53] In addition, neutrophils in SLE patients were found to have an active transcription signature and to be capable of interacting with deposited immune complexes (i.e., Fc region of IgG), which include C1q/anti-C1q, as well as contributing to complement activation.[54–56]

Our study focused on the cellular response in PMBCs downstream of anti-C1q. We found that C1q/anti-C1q complexes induce a pro-inflammatory cytokine response – TNF, IFNγ, and IL-10 – in PBMCs in a setting of unspecific aseptic inflammation. Moreover, CD154-mediated CD40 signaling in monocytes was discovered to be involved in the C1q/anti-C1q related increase in TNF.

Generally, autoantibodies and immune complexes are believed to be the primary drivers of SLE. However, aberrant cytokine levels, such as IL-6, IL-10, IL-17, TNF, and IFNγ, are commonly observed in SLE patients.[57–62] Besides their effects on differentiation, maturation, and activation of immune cells, cytokines are involved in local inflammatory responses and tissue injury. Additionally, an array of cytokines can be used to monitor disease activity and predict disease severity.[58] In the context of LN, abundant levels of TNF, IL-10, and IFNγ, as well as a simultaneous accumulation of anti-C1q in kidneys of SLE patients with renal involvement, have been observed, suggesting local synthesis of these particular cytokines.[21,49,63–65] In line with previous studies on C1q/anti-C1q complexes and their immunological effects on HMDMs,[32] we found a C1q/anti-C1q mediated increase in TNF and IFNγ in PBMCs of healthy donors after unspecific mild T cell activation. In contrast to HMDMs, IL-10 was also elevated in our experimental setting and reflected the situation in the serum of SLE patients with active disease.[66,67]

Concerning peripheral tolerance, T cell dysregulation is described as important in forming autoantibodies and the pathogenesis of SLE in general.[68] Expression of surface C1q receptors (i.e., gC1qR, cC1qR) in T cells suggests the capability to interact with C1q, which may affect T cell functions directly.[33,35,38] In fact, data from previous studies show immunoregulatory effects, such

as reduced proliferation, activation, and effector functions, upon C1q binding.[33,35,38] Interestingly, the binding of the collagen-like and globular heads region to their respective receptors is described as responsible for C1q's effects on T cells.[33,35,38] Contrary to earlier findings, our data on the direct impact of C1q on T cells do not demonstrate the same immunoregulatory effects. However, the experimental settings in the mentioned studies differ fundamentally. Our *in vitro* model of anti-C1q mediated autoimmunity uses small amounts of surface-bound C1q, whereas models used in previous studies investigated large amounts of soluble C1q in the cell culture medium. This important difference was introduced in our study to overcome two major challenges. First, allowing anti-C1q to bind C1q and thus enable the formation of immune complexes that require the exposure of cryptic epitopes being exposed on bound C1q.[69] Second, avoiding the potential interaction of soluble C1q with aggregated stimulating anti-CD3/CD28 antibodies could neutralize the activator and thus lead to misleading results. Interestingly, a study on T cells from C1q-deficient autoimmune-prone mice supports our observations regarding the proliferation and activation of human T cells.[70]

The binding of CD154 and its receptor CD40 are crucial for adaptive immunity and the pathogenic processes observed in SLE, including B cell proliferation and differentiation.[47] Furthermore, both surface molecules represent promising therapeutic targets, as shown by the recent developments of the inhibiting anti-CD154 Fab dapirolizumab pegol and the anti-CD40 antibody iscalimab, both being in clinical phase II and phase III trials, respectively.[71,72] Mostly described as a co-stimulatory factor in B cells, CD40 is a potent pro-inflammatory signaling pathway in monocytes and macrophages capable of inducing the synthesis of TNF and IL-1β.[43,73] These findings concur well with the induction of TNF in our co-culture experiments with monocytes and CD154 expressing RD cells and the decreased secretion of TNF upon CD154 inhibition. We could determine that cell–cell contact between C1q/anti-C1q primed monocytes and activated T cells is crucial and largely dependent on CD40–CD154 ligation for inflammatory cytokine secretion.

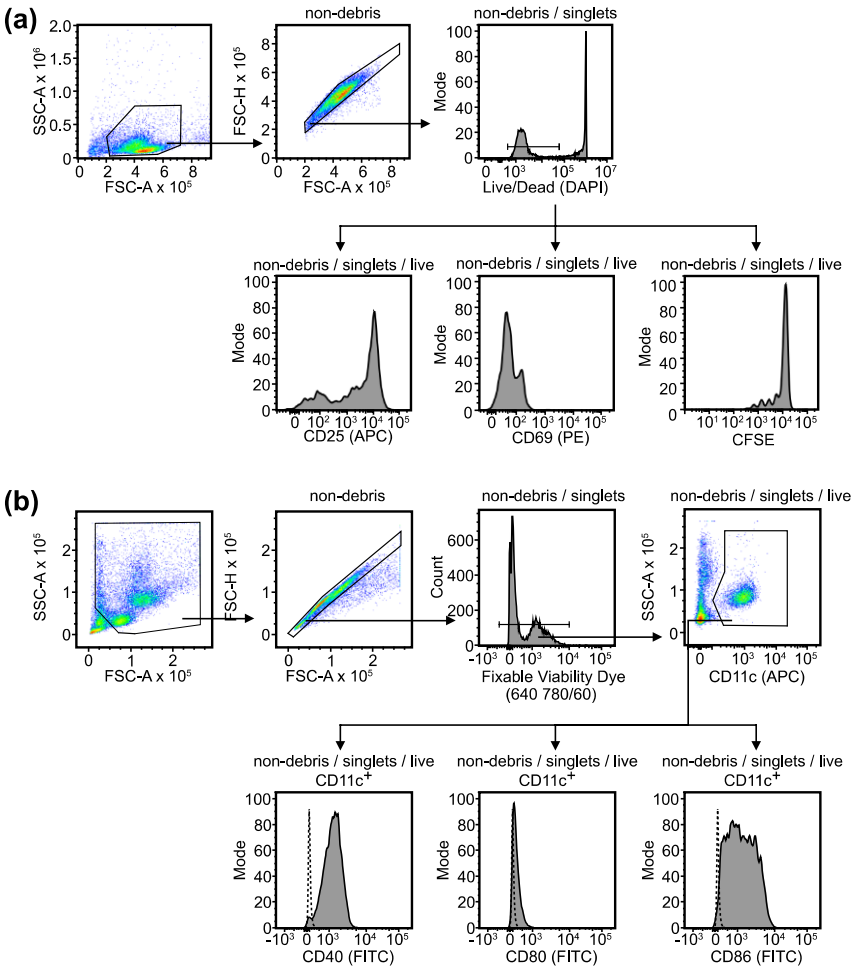
Contrary to expectations, CD40 surface expression in $CD11c^+$ cells was slightly decreased in the presence of C1q/anti-C1q compared to exposure to C1q alone. Previous studies show reduced CD40 expression and synthesis of pro-inflammatory cytokines in monocytes in the presence of IL-10 and IL-4.[74,75] Therefore, we hypothesize that this downregulation of CD40 is the result of a negative feedback mechanism caused by the increased IL-10 levels observed in our model. Next, we sought to investigate the responsible intracellular pathways in monocytes leading to TNF induction. CD40 signaling in monocytes is complex and comprises several pathways, including

TRAF-dependent and independent (i.e., JAK3-STAT5) pathways.[47] In line with previous studies, we describe two partially redundant signaling pathways, TRAF6 and JAK3-STAT5, responsible for TNF secretion downstream of C1q/anti-C1q.[48,76]

We are well aware that our study has some limitations. The first is the simplified *in vitro* model used in our study, which is likely to only partially reflect the complex events *in vivo,* including the possible role of other immune cells (i.e., neutrophils, B cells). Notably, the surface characteristics of a tissue culture treated plate to which C1q was attached to enable anti-C1q binding are probably different from biological surfaces. Furthermore, C1q's conformation closely depends on the target structure, affecting the exposure of neo-epitopes that allow anti-C1q binding.[77] However, plate-bound C1q allows anti-C1q binding that correlates with disease activity, as determined in many clinical studies.[17,78,79] In addition, our study on anti-C1q induced cytokine secretion is well in line with findings of the previous human *in vitro* studies, as well as the cytokine profile found in serum and kidney samples of patients with active SLE.[32,58] Additionally, instead of monoclonal anti-C1q, we used polyclonal high-affinity patient-derived anti-C1q antibodies for our analyses. Lastly, our *in vitro* model replaced the toll-like receptor 4 stimulant lipopolysaccharide with CD3/CD28 targeting antibody complexes. Not only does this adaptation result in an aseptic inflammatory setting, but it also shows that active T cells can trigger anti-C1q mediated inflammatory pathways.

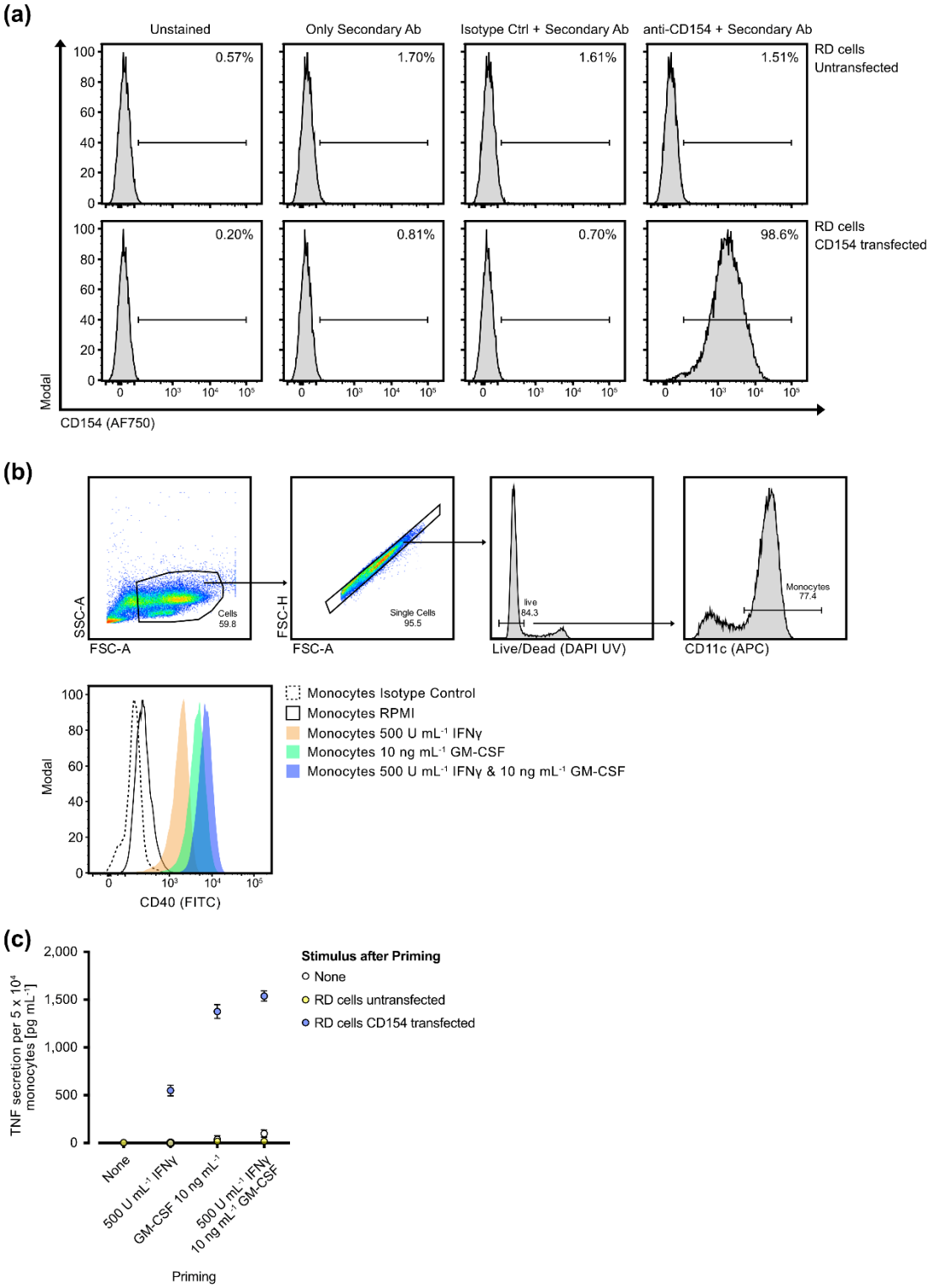
In conclusion, in this study, we describe the immunological effects of anti-C1q on PBMCs that depend on unspecific T cell activation. Our findings reveal that C1q/anti-C1q complexes upregulate TNF, IFNγ, and IL-10. TNF and IFNγ secretion from monocytes requires direct interaction with T cells, whereas IL-10 secretion from monocytes depends on further signals not provided in the co-culture of monocyte and T cell. Most notably, CD40 signaling in C1q/anti-C1q primed monocytes is essential for TNF production and could serve as a therapeutic target for anti-C1q mediated inflammation.

Supplementary Information Part I



Supplementary figure 1 Part I | Flow cytometry gating strategies

(a) Gating strategy for activation markers and proliferation in isolated T cells after 24 and 96 h of stimulation with anti-CD3/CD28 complexes, respectively. **(b)** CD11c$^+$ cell gating in PBMCs for expression levels of co-stimulatory surface markers after 24 h of stimulation with anti-CD3/CD28 complexes. Flow cytometry dot plots and histograms show one donor representative for six healthy donors.

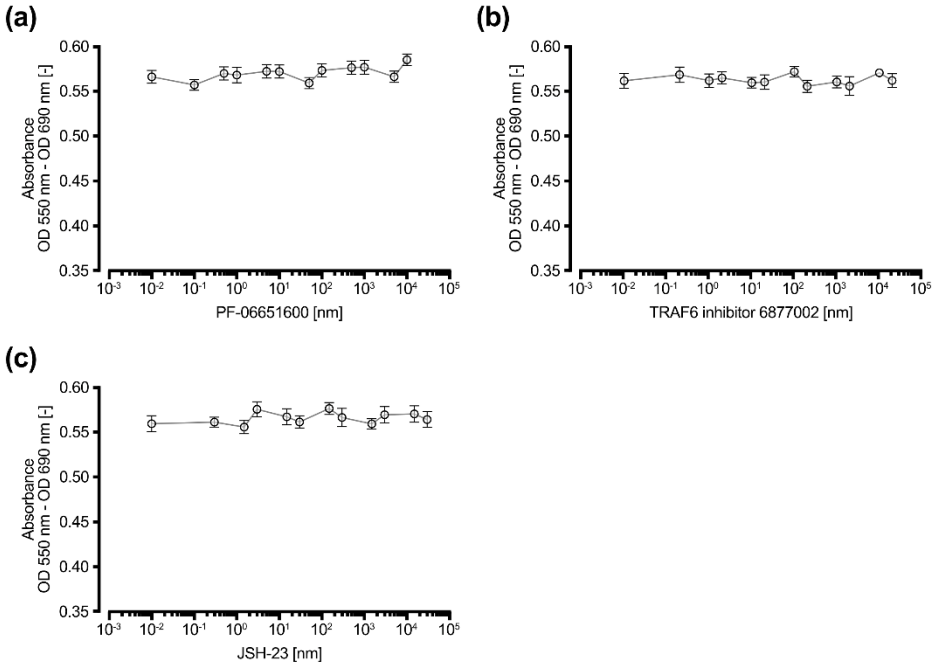


Supplementary figure 2 Part I | Expression of CD154 and CD40 in RD cells and monocytes, respectively

(Figure legend continues on next page)

(Continuation of Supplementary figure 2 Part I)

(a) CD154 expression in untransfected and CD154-transfected RD cells was analyzed using flow cytometry. Flow cytometry histograms display one experiment representative for three independent experiments. **(b)** After 18 h of stimulation with 500 U mL^{-1} IFNγ, 10 ng mL^{-1} granulocyte-macrophage colony stimulating factor (GM-CSF), or both, the expression of CD40 in monocytes was measured using flow cytometry. Flow cytometry dot plots and histograms display one donor representative for four healthy donors used to obtain monocytes. **(c)** To determine the priming condition for monocytes eliciting comparable TNF levels as seen in PBMCs, the monocytes were primed with 500 U mL^{-1} IFNγ, 10 ng mL^{-1} GM-CSF, or both for 18 h prior the addition of untransfected or CD154-transfected RD cells an additional 24 h. TNF secretion was assessed by ELISA. The data points with error bars represent the mean ± SEM of four healthy donors used to obtain monocytes.



Supplementary figure 3 Part I | Effect of CD40 signaling inhibitors on cell viability

The monocytes were isolated from PBMCs of healthy donors and cultured in the presence of C1q/anti-C1q. First, the cells were preincubated with 500 U mL^{-1} IFNγ for 18 h. Next, the monocytes were treated with **(a)** PF-06651600 0–10 μM (JAK3 inhibitor), **(b)** TRAF6 inhibitor 6877002 0–20 μM, **(c)** JSH-23 0–30 μM (NFκB inhibitor) for 24 h. Cell viability was assessed using a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data points with error bars represent mean ± SEM of six healthy donors used to obtain monocytes.

Part II

C1q-mediated *de novo* synthesis of C1q in HMDMs

Abstract

Objectives

C1q and anti-C1q are widely considered to be involved in the development of SLE. In circulation, C1q is predominantly associated with its serine proteases to form the C1 complex. However, the *in situ* production and release of C1q by myeloid cells was proven to be the primary source of C1q deposition in the glomeruli of LN patients. Additionally, this process likely promotes the formation of C1q and SLE patient-derived anti-C1q complexes (C1q/anti-C1q). On the other hand, C1q and C1q/anti-C1q can induce C1q synthesis in HMDMs. Thus, the aim of this study is to explore the mechanisms of C1q/anti-C1q-mediated C1q secretion in HMDMs.

Methods

In this study, the HMDMs of healthy donors were exposed to C1q or complexes of C1q and SLE patient-derived anti-C1q (C1q/anti-C1q). To better explain the consequences of C1q synthesis in HMDMs, differences in *C1QA* mRNA were evaluated using real-time quantitative polymerase chain reaction (qPCR), and the effects of the specific inhibition of transcription, translation, and secretion on C1q synthesis using a C1q-specific ELISA. Furthermore, the continuity of the C1q-releasing HMDM phenotype was challenged by reseeding experiments. Finally, C1q was labeled with biotin to validate the origin of the protein measured in the ELISA.

Results

The real-time qPCR results of *C1QA* mRNA did not reflect the differences observed in the amount of C1q in the cell culture medium. Additionally, the inhibition of protein synthesis and secretion machinery did not significantly affect the C1q levels observed in the cell culture medium. In contrast to previous studies, HMDMs did not continuously produce C1q after the removal of the C1q or C1q/anti-C1q coating. Furthermore, experiments with biotin-labeled C1q revealed that a significant fraction of the observed C1q in the cell culture supernatant originated from the coating itself, necessitating a new scientific question about how anti-C1q enhanced the detachment of C1q.

Conclusion

Overall, the presented work contradicted previous research on the C1q-inducing potential of C1q/anti-C1q complexes in HMDMs, necessitating further research.

Introduction

Complement has a significant impact on many human health conditions and diseases, highlighted by the search for new complement-targeting therapeutics.[162,163] Regarding self-tolerance and autoimmunity, deficiencies in complement proteins involved early in the complement cascades (i.e., C1q, C1r, C1s, and C4) have been described as detrimental.[2,4] Missing or aberrant activation of the classical pathway of complement is associated with SLE.[164] Especially in the case of C1q, numerous studies have made strong arguments linking C1q to SLE. First, hereditary homozygous C1q deficiency is strongly related to the development of SLE.[3,5] Second, patients with active SLE often present low levels of complement in serum, including both C1q itself and C4, C2, and C3. Last, one third of unselected patients with SLE and more than 90% of patients with active LN develop anti-C1q that are an established clinical biomarker for disease activity and a good predictor for LN.[91–94]

In biopsies of patients with LN, the deposition of C1q is a typical histological finding in the renal subendothelial space and along the glomerular basement membrane.[11,95,96] Unlike most proteins involved in the complement cascade, most of C1q is of non-hepatic origin.[17] Instead, C1q synthesis generally occurs locally by myeloid cells (e.g., macrophages, and DCs) in tissues. This idea is supported by a previous study showing no association between serum and glomerular C1q, suggesting infiltrating immune cells to be the primary source of glomerular C1q.[11] Additionally, biopsies of LN patients revealed elevated mRNA levels of C1q and cells capable of producing C1q associated with poor disease outcome.[165,166] This *in situ* biosynthesis of C1q facilitates the specific regulation of local inflammation, meaning the processing of immune complexes and regulation of innate and adaptive immunity.[167–169] In healthy individuals, the clearance of immune complexes occurs rapidly and heavily depends on complement.[27,170] After the binding of C1q to the immune complex, both C3b and C4b are deposited on the immune complex and are recognized by red blood cells, which are ultimately removed by macrophages and Kupffer cells in the spleen and liver, respectively.[27,170] If the clearance of immune complexes fails, these immune complexes often passively accumulate in the basement membrane of small blood vessels, such as the glomerulus in the kidney.[27,170] However, current data has suggested *in situ* formation rather than passive accumulation of glomerular immune complexes.[171,172] Additionally, inadequate complement activation in the presence of anti-C1q can lead to a high influx of immune cells and can increase complement consumption, causing inflamed tissue and organ damage.[97]

Anti-C1q are regarded as an essential but insufficient factor contributing to LN because renal involvement is unlikely in their absence.[94] For instance, anti-C1q accumulate in the glomeruli (50-fold higher concentration than that in serum) of patients with LN, ultimately facilitating the formation of C1q/anti-C1q complexes.[10,11] Regarding the regulation of C1q synthesis, Thanei *et al.* studied the effect of C1q/anti-C1q complexes on C1q production in HMDMs and demonstrated an induced, continuous *de novo* C1q synthesis, which could play an important immune-modulatory role in SLE.[22] Additionally, these researchers suggested that locally produced C1q is quickly consumed within the tissue causing both beneficial and damaging effects on the clearance of apoptotic material and chronic inflammation, respectively. However, despite this general increase in C1q secretion, not all HMDMs contributed equally to the *de novo* synthesized C1q. Notably, HMDMs were categorized as either low, intermediate, or high C1q-producing HMDMs by ImageStream^X. In the C1q/anti-C1q-conditioned HMDMs, approximately 20, 30, and 50% of the cells expressed low, intermediate, and high levels of intracellular C1q, respectively. In contrast, only 20% of the HMDMs in the control (i.e., plate-bound HSA) and C1q conditions displayed high levels of intracellularly stored C1q. Hence, the heterogeneous population of HMDMs seems to consist of phenotypically distinct HMDMs regarding C1q secretion that are not distinguishable by classical M1-like and M2-like macrophage characteristics.[22] Even so, potential autocrine and paracrine effects of locally produced C1q on HMDMs and other immune cells (e.g., T cells, B cells), respectively, have yet to be analyzed.

Considering the large number of functions of C1q[38] and the association of anti-C1q with SLE disease manifestations[91–94], it is highly likely that anti-C1q have a disease-modifying effect. However, exactly how anti-C1q contribute to disease activity and LN remains unclear. Therefore, identifying the phenotypical differences between low and high C1q-producing HMDMs could provide valuable insights into the underlying pathologic mechanisms in SLE mediated by anti-C1q.

Hypothesis

Based on current knowledge about the numerous functions of C1q, especially with the anti-inflammatory capabilities in HMDMs, this study demonstrated that the presence of C1q/anti-C1q complexes increases the C1q-mediated *de novo* synthesis of C1q in HMDMs.[22] We hypothesized that this effect resolves inflammation in the immediate vicinity and counteracts the increased complement consumption. From the work of Thanei *et al.*[22], I sought to better understand the biosynthesis of C1q triggered by C1q/anti-C1q complexes in low, intermediate, and high C1q-

producing HMDMs in the context of SLE. Furthermore, I aimed to explore the potential autocrine and paracrine effects of newly synthesized C1q on immune cells.

Methods

Isolation and differentiation of HMDMs

First, PBMCs were obtained from buffy coat preparations (Blood Transfusion Center of the University Hospital Basel, Basel, Switzerland) by density gradient centrifugation using Lymphoprep (Stemcell Technologies). Next, CD14 positive monocytes were isolated by immunomagnetic positive selection (CD14 MicroBeads, human; Miltenyi Biotec). Cells (yielding an average purity of 95–98% viable $CD14^+$ cells in our experiments as determined by flow cytometry) were then cultured in Dulbecco's modified essential medium (DMEM) supplemented with 3.97 mM GlutaMAX™, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin (all from Life Technologies), and 10% (v/v) NHS (pool of 60 healthy donors). Cells were kept at 37°C in 5% CO_2 for six to seven days to differentiate. After differentiation, the HMDMs were collected in PBS 5 mM ethylenediaminetetraacetic acid (EDTA) and resuspended in DMEM at a concentration of 500,000 cells mL^{-1} for later use in the *in vitro* anti-C1q autoimmunity model.

Anti-C1q / IgG source

All sera and plasma from SLE patients were provided by the SSCS. The patients fulfilled at least three of the 11 criteria of the ACR,[154,155] and were positive for anti-C1q (100–1,000 AU). Additionally, the SSCS is approved by Swiss Ethics covering the use of blood samples included in this study (Swiss Ethics 2017-01434).

In vitro anti-C1q autoimmunity model

The *in vitro* model of anti-C1q autoimmunity was used as described before.[16,22,158] Briefly, flat-bottom 96-well plates (Eppendorf, Hamburg, Germany) were coated with 70 µL of 5 µg mL^{-1} purified human C1q (Complement Technology, Tyler, Tx, USA) and 5 µg mL^{-1} HAS in coating buffer (0.4 M sodium carbonate buffer; pH 9.6) overnight at 4°C. Next, the plates were washed twice with 140 µL PBS. Subsequently, each serum sample was centrifuged at 14,000 *g* at 4°C for 30 min and diluted at 1:100 in PBS 1 M NaCl before the addition of 70 µL of diluted sample and incubation on a shaker (500 rpm) at room temperature for 1 h. Again, plates were washed four times with 140 µL PBS. Prior to stimulation, HMDMs (50,000 $well^{-1}$) were added to 100 µL cell culture medium and allowed to adhere for 30 min. Stimulation with 10 ng mL^{-1} lipopolysaccharide (LPS, Sigma-Aldrich) occurred in a final volume of 200 µL lasted 22 h.

C1q quantification

After 22 h of cell culture, the cell culture supernatant was quantified for C1q by a sandwich ELISA as described previously.[22] In brief, a human C1q-specific mouse mAb (clone 34A4)[100] was coated overnight at 4°C in coating buffer (0.4 M sodium carbonate buffer, pH 9.6). To avoid unspecific binding, all unoccupied binding sites were blocked with assay diluent (PBS containing 3% [m/v] BSA). As a standard curve, purified human C1q was added in eight serial dilutions covering a range of 0–25 ng mL^{-1} in assay diluent. Following the incubation of undiluted samples and standards for 2 h at room temperature, a goat anti-human C1q polyclonal antibody (Quidel) combined with an HRP-conjugated mouse anti-goat IgG antibody (Sigma-Aldrich) was diluted in assay diluent and added in succession for 1 h each at room temperature. Finally, the C1q concentration was assessed by measuring enzyme activity after the addition of TMB substrate. Next, concentrations were calculated using a sigmoidal four-parameter model. The lower limit of detection was 0.01 ng mL^{-1}.[22]

Gene expression analysis

The RNA of 200,000 HMDMs was isolated using a NucleoSpin RNA kit (Macherey Nagel, Düren, Germany). Subsequently, reverse transcription was achieved with GoScript Reverse Transcriptase with oligo(dT) (Promega, Madison, WI, USA). Finally, real-time qPCR was performed with GoTaq PCR Master Mix (Promega) on a Viia 7 real-time qPCR system (Thermo Fisher). All kits were used according to the manufacturer's instructions. Analyzed primer sequences (Microsynth AG, Balgach, Switzerland) are listed in Table 3. Beyond this, relative gene expression was calculated using the comparative CT method[173] with the geometric mean of *B2M* (encoding β-2-microglobulin) and *GAPDH* (encoding glyceraldehyde 3-phosphate dehydrogenase) as a reference (identified as stably expressed genes by geNorm[174] in Supplementary figure 1 Part II, page 69).

Table 3 | Primer sequences used for quantitative real-time qPCR

Gene	5' forward	3' reverse	Ref
C1QA exon 3	TGG AGT TGA CAA CAG GAG GC	CGA TAT GGC CAG CAC ACA GA	175
C1QB exon 1–2	GAC CGA GGG CAG TAG GCT C	TCA TCA TAC TGT GTC AGA CGC C	175
C1QC exon 2–3	AAG GAR GGG TAC GAC GGA	GTA AGC CGG GTT CTC CCT TC	175
GAPHD	CGG AGT CAA CGG ATT TGG TCG	TCT CGC TCC TGG AAG ATG GTG AT	176
B2M	TCT CGC TCC GTG GCC TTA	GGA GTA CGC TGG ATA GCC TC	177

Inhibition of de novo C1q synthesis and secretion

Various concentrations of actinomycin D (1–50 μg mL^{-1}), cycloheximide (1–300 μg mL^{-1}), and brefeldin A (0.1–10 μg mL^{-1}) dissolved in DMSO (final DMSO concentration in the cell culture medium ≤ 1% [v/v]) were added to HMDMs in the *in vitro* anti-C1q autoimmunity model 30 min before the stimulation with 10 ng mL^{-1} LPS.

Biotin labeling of C1q

The covalent linking of biotin to purified human C1q was performed according to the manufacturer's instructions (EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit, Thermofisher). In brief, the biotin labeling reagent was freshly prepared in ultrapure water (Life Technologies) yielding a 10 mM stock solution. For the coupling reaction, a 20-fold excess of biotin labeling reagent was added to C1q for 2 h at 4°C. To eliminate excessive uncoupled biotin, the mixture was loaded onto a centrifugal filter for protein purification with a molecular cut-off of 3 kDa (Amicon® Ultra 3K device, Merck, Darmstadt, Germany) and centrifuged for 30 min at 14,000 *g* and 4°C. Ultimately, the success of the biotin labeling was confirmed in an adapted version of the previously described C1q ELISA protocol using a streptavidin-HRP (1:5,000; Sigma-Aldrich) conjugate to detect C1q-biotin.

Statistical analysis

First, the normal distribution of the data was confirmed by the Shapiro–Wilk test. Parametric statistical analyses involving more than two groups were performed using one-way ANOVA with repeated measurements including Bonferroni's correction. Next, comparisons of multiple groups at different times were determined using two-way ANOVA. Statistical significance was considered

with $*P \leq 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. Analyses were conducted with GraphPad Prism 9.1.2.

Results

C1q/anti-C1q complexes increase C1q concentrations in cell culture supernatant

Previously, Thanei *et al.* described the *de novo* synthesis of C1q in HMDMs in an *in vitro* anti-C1q mediated autoimmunity model.[22] HMDMs exposed to C1q/anti-C1q complexes showed an increased production of newly formed C1q. To study the mechanisms involved in more detail, I first established the model with sera from three selected anti-C1q positive (cut-off 50 AU) SLE patients. Next, HMDMs were exposed to various plate-bound coatings, including HSA, C1q, and C1q/anti-C1q, the latter derived from the three different SLE patients (SLE1–3) with varying anti-C1q levels (SLE1: 1,000 AU anti-C1q; SLE2: 658 AU anti-C1q; SLE3 173 AU anti-C1q). To mimic the inflammatory state of macrophages observed in patients, a proinflammatory environment was created by the addition of 10 ng mL^{-1} LPS. After 22 h, the cell culture supernatants were analyzed for C1q by ELISA (Figure 14). In line with previous findings, C1q/anti-C1q complexes increased the amount of C1q found in the supernatants when compared with cells that were exposed to only C1q. This increase was significant for SLE1 ($P = 0.020$) and SLE2 ($P = 0.027$) but not for SLE3 ($P = 0.061$). Additionally, Thanei *et. al* discovered a correlation between anti-C1q levels and *de novo* C1q concentrations,[22] which I observed as well with a trend in SLE1–3.

The continuous biosynthesis of C1q was previously described in monocytes and HMDMs.[19,22] To evaluate the kinetics of C1q secretion in our *in vitro* anti-C1q mediated autoimmunity model, I compared C1q concentrations for the coatings HSA, C1q, and C1q/anti-C1q (derived from SLE1) after 0, 3, 6, and 22 h of LPS stimulation. Differences in C1q concentrations between C1q and C1q/anti-C1q were observed as early as 3 h of cell culture, whereas C1q synthesis on HSA began to increase at 6 h (Figure 14b). Furthermore, the linear kinetics in these experiments for all three coatings were consistent with previous research.[19,22]

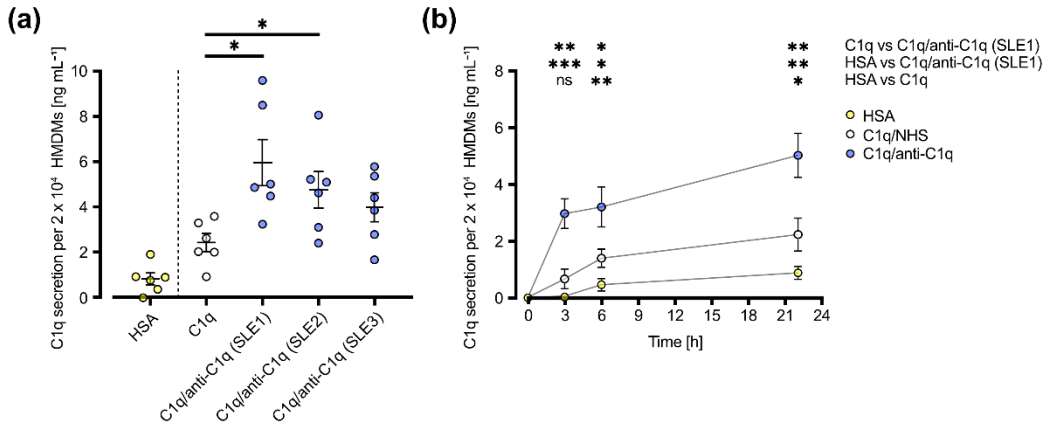


Figure 14 | Anti-C1q bound to plate-bound C1q increases C1q concentration in cell culture medium in presence of HMDMs

(a) HMDMs from six healthy donors were incubated on either HSA, C1q, or C1q/anti-C1q (SLE1–3) complexes (anti-C1q derived from three different SLE patients) and stimulated with 10 ng mL^{-1} LPS for 22 h. The data points represent mean concentrations of three replicates of one single individual and horizontal lines with error bars denote mean ± standard error of the mean (SEM) of all six individuals. Connecting lines linking each individual donor were omitted to refine readability of the figure. One-way ANOVA with repeated measurements including Bonferroni's correction (all vs C1q). *$P \leq 0.05$. **(b)** The kinetic of C1q measured in the cell culture supernatant over 22 h with HMDMs exposed to HSA, C1q, or C1q/anti-C1q coating. The data points with error bars represent the mean ± SEM of six healthy donors used to obtain HMDMs. Two-way ANOVA including Tukey's correction. ***$P < 0.001$, **$P < 0.01$, *$P \leq 0.05$, ns: not significant.

Transcription of C1q in HMDMs is not affected by C1q/anti-C1q complexes

To gain a better understanding of how C1q secretion is regulated in HMDMs, I next assessed the transcription of C1q. To achieve this goal, real-time qPCR analysis of all three C1q chains, namely *C1QA*, *C1QB*, and *C1QC,* in HMDMs was performed after 22 h of exposure to plate-bound HSA, C1q, and C1q/anti-C1q derived from three different SLE patients (SLE1-3). The overall fold change in mRNA for all three C1q chains did not increase significantly more in the presence of C1q/anti-C1q than in C1q alone. Moreover, the mRNA expression was independent of the anti-C1q source and levels (Figure 15a). Notably, *C1QC* demonstrated a significantly change in expression between the control coating HSA and C1q ($P = 0.015$).

To exclude time-dependent mRNA changes, I analyzed differences in *C1QA* mRNA between coatings after 3, 6, and 22 h of cell culture. Again, no significant changes in *C1QA* mRNA were observed between the three coatings (Figure 15b).

Overall, these results suggest an increase in C1q synthesis and release by HMDMs into the cell culture supernatant in the presence of C1q/anti-C1q. However, the observation, which was made on the protein level, was not reflected on a transcriptional level, as demonstrated by real-time qPCR.

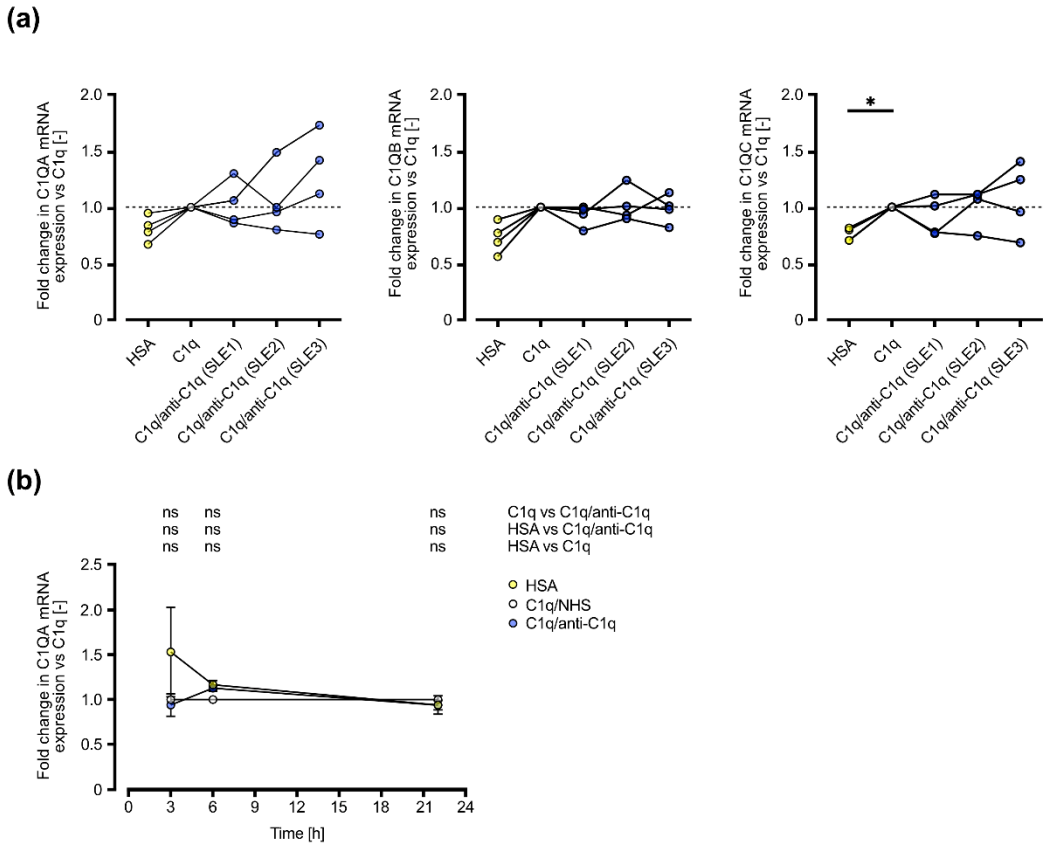


Figure 15 | Transcription of all three C1q chains in HMDMs not affected by exposure to different protein coatings

(a) Fold change in gene expression of *C1QA*, *C1QB*, and *C1QC* in HMDMs exposed to either HSA, C1q, or C1q/anti-C1q (SLE1–3) complexes (anti-C1q derived from three different SLE patients) for 22 h was analyzed by real-time qPCR. The data points represent four healthy donors from independent experiments. One-way ANOVA with repeated measurements including Bonferroni's correction (all vs C1q). $*P \leq 0.05$. **(b)** *C1QA* mRNA expression pattern in HMDMs was measured by real-time qPCR after 3, 6, and 22 h of exposure to either HSA, C1q, or C1q/anti-C1q (SLE1). The data points with error bars denote mean ± SEM of four healthy donors from independent experiments. Two-way ANOVA including Tukey's correction. ns: not significant.

Specific inhibition of transcription, translation, and secretion apparatus does not alter C1q concentrations in cell culture supernatant

Because the differences in C1q levels in the supernatants between the plate-bound coatings HSA, C1q, and C1q/anti-C1q complexes were not reflected in the *C1QA* mRNA, I next addressed whether the biosynthesis of C1q in HMDMs is regulated at a later stage of protein synthesis (i.e., translation, secretion). To evaluate all stages of C1q synthesis, specific inhibitors to target transcription (actinomycin D), translation (cycloheximide), and secretion (brefeldin A) were added 30 min before the LPS stimulation for the entire 22 h of the cell culture. Next, I validated the inhibitory efficacy of actinomycin D, cycloheximide, and brefeldin A by measuring their potential to inhibit the secretion of LPS-induced TNF in HMDMs. All three compounds significantly reduced TNF secretion ($P < 0.001$) at the lowest chosen concentration, i.e. 1 μg mL^{-1} actinomycin D, 1 μg mL^{-1} cycloheximide, and 0.1 μg mL^{-1} brefeldin A (Supplementary figure 2 Part II, page 68). Notably, the quantification of C1q in the cell culture supernatant did not reveal a significant decrease in C1q after the inhibition of all three stages with various concentrations of the inhibitors (Figure 16). Control samples with 1% (v/v) DMSO and inhibitors demonstrated no effect on C1q secretion and cell viability, respectively (Supplementary figure 3 Part II, page 69).

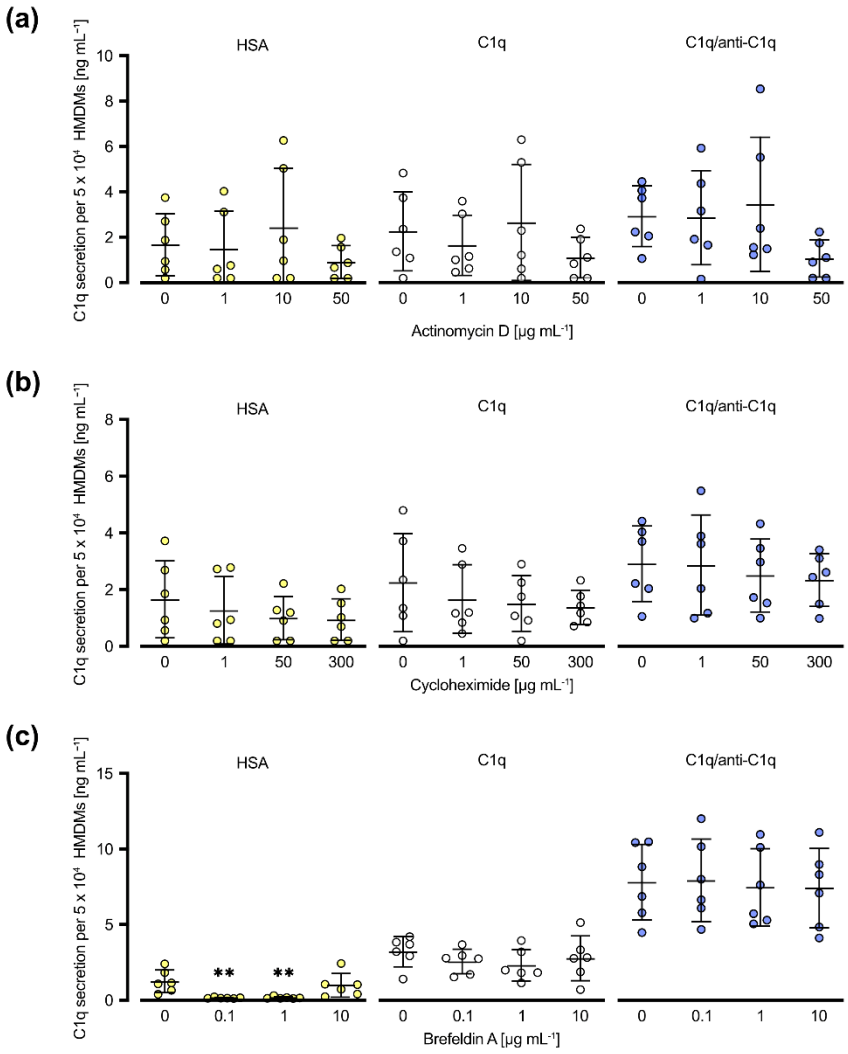


Figure 16 | C1q concentration in cell culture supernatants of HMDMs in anti-C1q-mediated autoimmunity model remains unaltered by inhibition of transcription, translation, or secretion machinery

The HMDMs of six healthy donors were exposed to either HSA, C1q, or C1q/anti-C1q with concomitant additions of various concentrations of **(a)** actinomycin D (1-50 µg mL^{-1}), **(b)** cycloheximide (1-300 µg mL^{-1}), or **(c)** brefeldin A (0.1-10 µg mL^{-1}) in independent experiments (donors in **[c]** differed from those in **[a and b]**). After 22 h of stimulation with 10 ng mL^{-1} LPS, cell culture supernatant was analyzed for soluble C1q using ELISA. Horizontal lines with error bars represent mean ± SEM. Connecting lines linking each individual donor were omitted to refine readability of the figure. One-way ANOVA with repeated measurements including Bonferroni correction (for each coating separately: all vs no inhibitor). $^{**}P < 0.01$.

C1q concentration in cell culture supernatants of HMDMs is not persistent after removal of priming condition

To assess whether the priming of the HMDMs by plate-bound coating was persistent, I collected the cells after the initial 22 h of cell culture, washed, and reseeded them in new uncoated cell

culture plates. The cumulative C1q concentration in the cell culture supernatant were analyzed after 48, 96, and 144 h after reseeding. After 48 h of reseeding time, the C1q concentration decreased significantly. This effect remained for after 96 as well as 144 h (Figure 17). Nevertheless, the initial differences after 22 h of priming were still significant in our experiments indicating a non-sustainable C1q secretion.

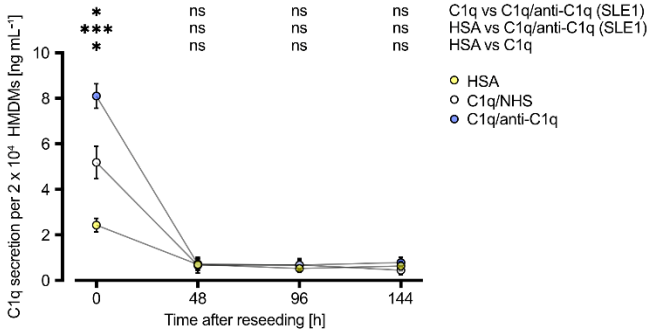


Figure 17 | C1q concentration in cell culture supernatants of HMDMs not persistent after reseeding cells in fresh uncoated cell culture plates

HMDMs were incubated on either HSA, C1q, or C1q/anti-C1q and stimulated with 10 ng mL^{-1} LPS. After 22 h of cell culture (indicated 0 h after reseeding in the figure), the cells were harvested, washed, resuspended in a fresh cell culture medium, and reseeded in new wells without protein coatings. Next, the supernatants were collected at indicated time points (48, 96, and 144 h) and analyzed for soluble C1q using ELISA. The data points show cumulative C1q concentration of four healthy donors (mean ± SEM) from independent experiments. Two-way ANOVA including Tukey's correction. ***$P < 0.001$, *$P \leq 0.05$, ns: not significant.

Biotin labeling of C1q reveals majority of C1q observed in cell culture medium originates from coating

Next, I aimed to identify the source of C1q in the supernatant after unsuccessfully trying to inhibit its *de novo* biosynthesis at different stages (i.e., transcription, translation, secretion). For this purpose, I covalently labeled the C1q with biotin which was then used to coat the cell culture plates. The binding of anti-C1q to C1q-biotin was confirmed by an anti-C1q ELISA, revealing no differences in the amount of bound anti-C1q between C1q and C1q-biotin (Supplementary figure 4 Part II, page 69). Analogously to previous experiments, HMDMs were exposed to five different plate-bound coatings including HSA, C1q, C1q-biotin, C1q/anti-C1q, and C1q-biotin/anti-C1q. After 22 h, the cell culture supernatants were analyzed using an ELISA specific to measure C1q (Figure 18a) and C1q-biotin (Figure 18b) concentrations. Notably, C1q-biotin produced a significant higher C1q concentration in the supernatant than native C1q in both the presence (P = 0.010, C1q vs C1q-biotin) and absence (P = 0.044, C1q/anti-C1q vs C1q-biotin/anti-C1q) of anti-C1q (Figure 18a). However, the majority of the C1q present in the supernatant was biotinylated,

as revealed by the biotin-specific ELISA (Figure 18b). These results demonstrate the fact that a significant amount of the C1q in the supernatant originated from the plate and was thus not newly synthesized by the HMDMs.

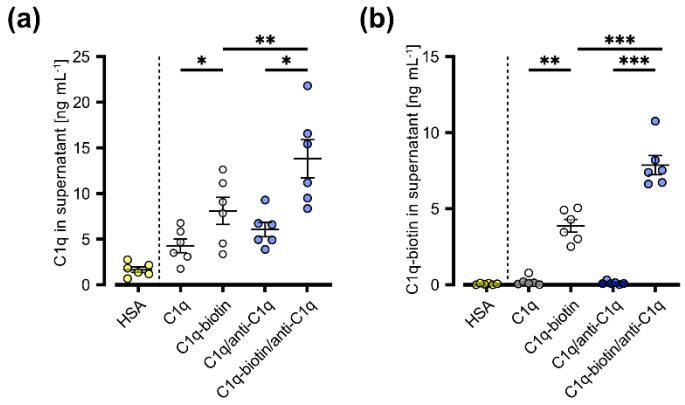


Figure 18 | Majority of soluble C1q originates from initial coating

HMDMs were incubated on either C1q, C1q-biotin, C1q/anti-C1q, or C1q-biotin/anti-C1q for 22 h with concomitant LPS stimulation. To analyze the amount of C1q and C1q-biotin, the cell culture supernatant was analyzed using a **(a)** C1q or **(b)** C1q-biotin specific sandwich ELISA, respectively. The data points represent six healthy donors with horizontal lines and error bars indicating mean ± SEM. Connecting lines linking each individual donor were omitted to refine readability of the figure. One-way ANOVA with repeated measurements including Bonferroni's correction. $***P < 0.001$, $**P < 0.005$, $*P \leq 0.05$.

Discussion

In the clearance of apoptotic cells and immune complexes, C1q is an indispensable factor.[27,170] By interfering with C1q's functions, anti-C1q are believed to have a pathogenic role in the development and exacerbation of LN and SLE in general. In both acute and upcoming flares, anti-C1q levels increase and correlate well with disease activity and renal involvement.[10,11] The occurrence of anti-C1q in SLE is often accompanied by low serum levels of complement (i.e., C1q, C4, and C2)[81–84] and the accumulation of C1q in the glomeruli of LN patients suggesting local biosynthesis and secretion[11,95,96] However, the exact pathologic mechanisms of LN remain unclear.

This study focused on the potential of C1q and C1q/anti-C1q complexes to induce *de novo* synthesis and release of C1q in HMDMs. As previously reported[22], I found increased C1q levels in the cell culture medium of HMDMs. However, the inhibition of protein synthesis (i.e., transcription and translation) and secretion indicate that C1q is likely not freshly synthesized in HMDMs in the experimental setting used to obtain these data. Furthermore, the modification of C1q through biotin labeling revealed plate-bound C1q to be the primary source of the soluble C1q measured in the cell culture medium.

Tissue-resident myeloid cells, such as macrophages and DCs, are described as the predominant source of endogenous C1q.[17] In circulation, most of the C1q is complexed with the two serine proteases C1r and C1s to form C1. The presence of free C1q is limited to local synthesis by resident myeloid cells in affected tissue.[50] Multiple regulating factors are involved in the secretion of C1q, including the differentiation state of the myeloid cell, presence of cytokines (e.g., IL-1β, IFNγ, and IL-6), and several endogenous (e.g., immune complexes) and exogeneous (e.g., lipopolysaccharide, C3b-opsonized zymosan, acetylsalicylic acid, and methylprednisolone)[19,178–181] compounds. Regarding the differentiation state of the cells, in this study by Kaul *et al.*, the monocytes failed to substantially participate in the secretion of C1q (despite detectable C1q mRNA).[181] On the other hand DCs and macrophages actively produced and secreted C1q into their surroundings.[12,22,182] Notably, the presentation of exogeneous purified soluble and plate-bound C1q to murine and human macrophages, respectively, caused an autocrine induction of C1q synthesis.[12,22,183] In another study published by our group, this C1q-mediated C1q production was enhanced in the HMDMs in the presence of anti-C1q forming C1q/anti-C1q.[22] In this scenario, the elevated C1q secretion was proposed to be an attempt of the HMDMs to counter-act the insufficient C1q levels, which are quickly consumed, and the inflammatory effect of the antibody. On the one hand, this observation could be considered beneficial in the context of

phagocytosis and the clearance of immune complexes and apoptotic cells. On the other hand, newly deposited C1q could be recognized by the highly abundant anti-C1q, resulting in a vicious circle accompanied by complement activation and inflammation.[22]

In line with previous studies on C1q-mediated C1q production, C1q concentrations were increased more when HMDMs were exposed to C1q and C1q/anti-C1q than in the control protein HSA (Figure 14a). Additionally, a time-dependent analysis of C1q levels revealed a steep rise within the first three hours of the cell culture and a more linear growth afterwards for the remaining time points (Figure 14b). However, in contrast to previous results, the C1q secretion observed was not sustained after reseeding the HMDMs in uncoated plates, suggesting no or a only transient induction of C1q synthesis (Figure 17).[22]

Although previous studies on C1q synthesis had detected variations in C1q mRNA levels between different conditions (in both the absence and presence of exogeneous C1q),[183,184] the results presented in Figure 15 do not reflect the situation observed in this study at the protein level in the cell culture medium. Based on these results, the regulation of the C1q synthesis at a later stage, specifically translation and the subsequent secretion, was considered responsible for the C1q-mediated increase in soluble C1q. This hypothesis was supported by the fact that the inhibition of the transcription machinery by actinomycin D did not significantly impact C1q secretion (Figure 16a). In an early publication by Müller *et al.*, the addition of cycloheximide (0.5 $\mu g\ mL^{-1}$) of low concentrations caused a reversible and almost complete reduction in C1q synthesis (measured in cumulative C1 activity) in guinea pig macrophages.[185] Similarly, small amounts of brefeldin A (0.14 $\mu g\ mL^{-1}$) were reported by Bulla *et al.* to abolish C1q expression in decidual endothelial cells.[186] However, the results obtained from the inhibition experiments blocking the translation and secretion of *de novo* synthesized proteins with even high concentrations of cycloheximide (1–300 $\mu g\ mL^{-1}$) and brefeldin A (0.1–10 $\mu g\ mL^{-1}$), respectively, did not prevent C1q secretion, contradicting previous studies (Figure 16b and c).[185,186] Notably, HMDMs might behave differently when comparing to human decidual endothelial cells and guinea pig-derived macrophages.

In this vein, the findings here do not suggest *de novo* synthesis of C1q in HMDMs in the *in vitro* model used in this project. Specifically, the modification of the plate-bound C1q involving the addition of biotin to the lysine residues revealed that a majority of the detected C1q in the cell culture medium originated from the initial coating of the plate (Figure 18a and b). Beyond this, the biotin labeling could alter the biochemical and biophysical properties of C1q with subsequent conformational changes leading to weaker attachment of the globular heads to the cell culture

plate. However, these results suggest that previous observations on C1q-mediated synthesis of C1q should be questioned. Thus, further research is needed to finally assess the role of C1q as an autocrine inducer of C1q synthesis.

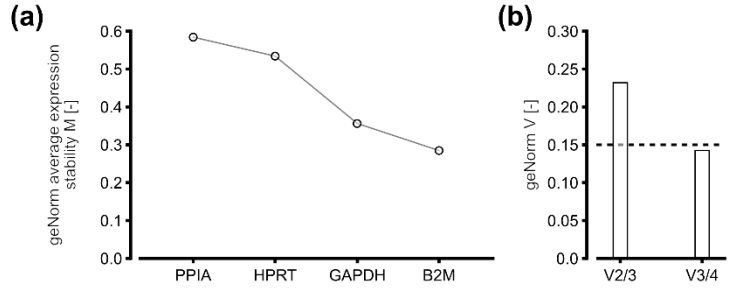
Several limitations affected this study. First, the complex pathological mechanisms at play *in vivo* in humans were significantly simplified and not adequately represented in the *in vitro* model. Infiltrating macrophages are allegedly correlated with disease progression in patients with LN and a major source of local C1q deposition in the glomeruli,[11] other immune cells present in the extravascular space of the kidney (e.g., B, T, NK, and DCs)[166] are likely to influence C1q production by infiltrating myeloid cells, further complicating the situation *in vivo*. However, analyzing single cell population produces more evident conclusions providing solid understanding of the basic mechanisms of C1q synthesis and could be applied on further experiments. Although I isolated and studied $CD14^+$ HMDMs, the monocyte differentiation was not directed towards M1-like or M2-like macrophages by adding granulocyte-macrophage colony-stimulating factor or macrophage colony-stimulating factor, respectively. This unguided differentiation of monocytes results in a heterogeneous population of HMDMs with both pro-inflammatory M1 and inflammation-resolving M2 macrophages expressing opposing phenotypes and likely a mixed response to C1q production. The cells used in this *in vitro* study were obtained from the blood of healthy donors and likely behaved differently than would cells from patients with SLE. However, studying the immunologic effects of C1q and C1q/anti-C1q in homogeneous healthy cells could provide valuable knowledge that could later be applied to cells derived from SLE patients. Additionally, the presentation of C1q on a plastic surface (i.e., the polystyrene cell culture plate) is not representative of the situation under physiological conditions with C1q being bound to one of its targets (e.g., apoptotic cells, immune complexes, pathogens). The conformation of C1q largely depends on the target structure of the pattern recognizing globular heads, impacting the binding of potential interacting receptors on effector cells, such as macrophages and DCs. Although a more physiological target of C1q is desirable, few to no fluctuations occurred in the presentation of C1q on the surface of a cell culture plate compared with apoptotic cells or immune complexes. This experimental setup facilitates a higher reproducibility between experiments. Furthermore, the quantification of anti-C1q is routinely performed with plate-bound C1q and the resulting anti-C1q levels quantified on this basis correlate with disease severity, as determined in many clinical studies.[91,93,94,187–190]

Therefore, future research shall address these limitations to further explain C1q and C1q/anti-C1q–mediated C1q production in HMDMs. To evaluate the extent to which C1q is freshly

secreted or originates from the prepared cell culture plate, future research could involve the generation of C1q deficient macrophages in either primary healthy donor-derived cells or a human macrophage cell line (e.g., THP-1), using clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 system. The appearance of C1q in the cell culture medium in the presence of C1q deficient cells would confirm the hypothesis that plate-bound C1q is detached. A more generalized top-down approach could include transcriptional and/or proteomic analyses of single immune cells present in biopsies from patients with LN. Ultimately, such an approach could provide valuable information on which cells are responsible for the *in situ* production of C1q *in vivo*. Additionally, this work could highlight the receptors and intracellular pathways involved in these processes. Alternatively, the same analyses could be applied to the HMDMs in the *in vitro* model.

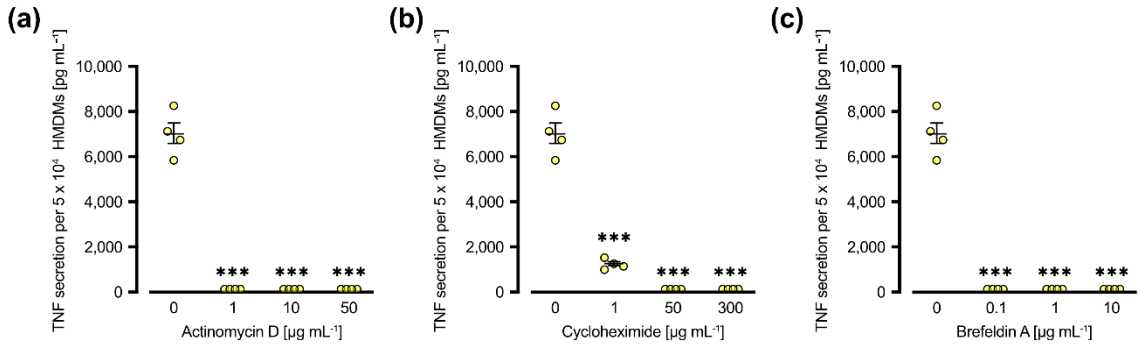
In closing, this study's findings do not support the previously proposed notion of *de novo* synthesis of C1q in HMDMs triggered by C1q and C1q/anti-C1q complexes.[22] Instead, these results indicate that HMDMs induce conformational changes in C1q that leads to the detachment and release of C1q into the cell culture medium. This effect is enhanced by the presence of anti-C1q. These results suggest further research is needed to assess C1q synthesis in HMDMs.

Supplementary Information Part II



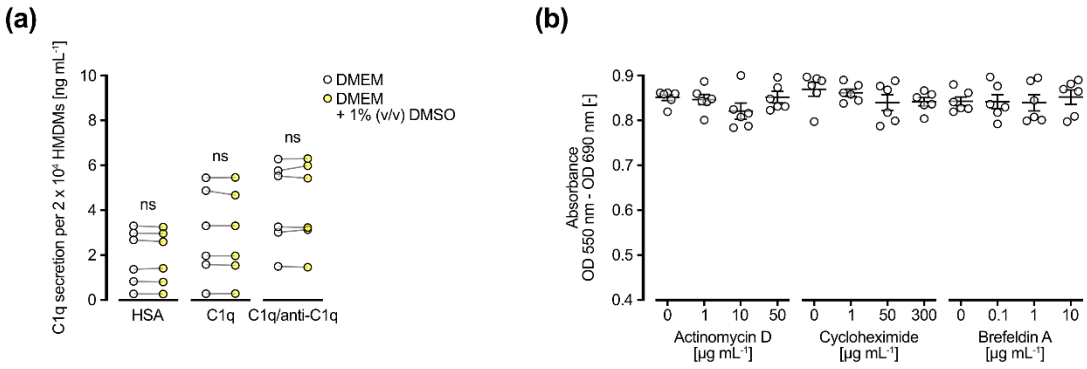
Supplementary figure 1 Part II | Selection of reference genes based on expression stability

(a) GeNorm M (lower M value represents more stable reference genes) results for *PPIA*, *HPRT*, *GAPDH*, and *B2M* in HMDMs exposed to HSA, C1q, and C1q/anti-C1q. **(b)** To evaluate combinations of reference genes, GeNorm V values were calculated for tested reference genes (V n/n+1).



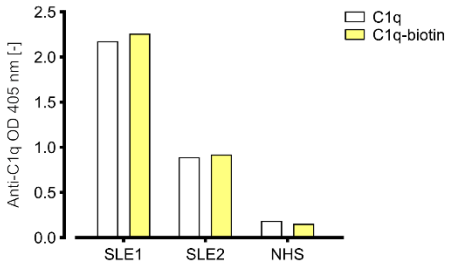
Supplementary figure 2 Part II | LPS-induced TNF secretion by HMDMs inhibited after addition of transcription, translation, and secretion specific inhibitors

HMDMs of four healthy donors were cultured with concomitant addition of various concentrations of **(a)** actinomycin D (1–50 µg mL^{-1}), **(b)** cycloheximide (1–300 µg mL^{-1}), and **(c)** brefeldin A (0.1–10 µg mL^{-1}). After 22 h of stimulation with 10 ng mL^{-1} LPS, cell culture supernatant was analyzed for TNF by ELISA. The data points represent four healthy donors from independent experiments. Horizontal lines with error bars represent mean ± SEM. Connecting lines linking each individual donor were omitted to refine readability of the figure. One-way ANOVA with repeated measurements including Bonferroni's correction (all vs no inhibitor). ***$P < 0.001$.



Supplementary figure 3 Part II | No effect of DMSO and inhibitors on C1q secretion and cell viability, respectively

HMDMs of six healthy donors were cultured with **(a)** 1% (v/v) DMSO and **(b)** various concentrations of actinomycin D (1–50 µg mL^{-1}), cycloheximide (1–300 µg mL^{-1}), and brefeldin A (0.1–10 µg mL^{-1}). After 22 h of stimulation with 10 ng mL^{-1} LPS, cell culture supernatant and cells were analyzed for **(a)** C1q and **(b)** cell viability using a C1q-specific ELISA and MTT assay, respectively. The data points represent six healthy donors from independent experiments. **(a)** Connecting lines link data points of a single individual. Paired *t*-test, ns: not significant. **(b)** Horizontal lines with error bars denote mean ± SEM. Connecting lines linking each individual donor were omitted to refine readability of the figure. One-way ANOVA with repeated measurements including Bonferroni's correction (all vs no inhibitor).



Supplementary figure 4 Part II | Binding of anti-C1q to C1q and C1q-biotin

To analyze differences induced by biotin labeling of C1q (C1q-biotin), binding of anti-C1q derived from sera of two patients with SLE and one NH (NHS) was evaluated using an anti-C1q specific ELISA. Bars represent mean of three technical replicates.

Part III

Epitope-specificity of anti-C1q autoantibodies in SLE

Contributions

In this part, my personal contributions are focused on the scientific discussions leading to the establishment of the experimental protocols, analyses conducted, reviewing and troubleshooting source code for the statistical analyses in R, and figure drafting for the manuscript. I was neither directly involved in the conduction of the experiments nor in the collection and interpretation of clinical data.

Abstract

Objective

In patients with SLE complement C1q is frequently targeted by autoantibodies, that correlate best with active renal disease. Anti-C1q bind to largely unknown epitopes on the CLR of this highly functional molecule. Here we aimed at exploring the role of epitope-specific anti-C1q in SLE patients.

Methods

First, 22 sera of SLE patients, healthy controls and anti-C1q positive patients without SLE were screened for anti-C1q epitopes by a PEPperMAP® microarray, expressing CLR of C1q-derived peptides with one AA shift in different lengths and conformations. Afterwards, samples of 378 SLE patients and 100 healthy blood donors were analyzed for antibodies against the identified epitopes by peptide-based ELISA. Relationships between peptide-specific autoantibodies and SLE disease manifestations were explored by logistic regression models.

Results

The epitope mapping showed increased IgG binding to three peptides of the C1q A- and three of the C1q B-chain. In subsequent peptide-based ELISAs, SLE sera showed significantly higher binding to two N-terminally located C1q A-chain peptides than controls ($P < 0.0001$), but not to the other peptides. While anti-C1q were associated with a broad spectrum of disease manifestations, some of the peptide-antibodies were associated with selected disease manifestations, and antibodies against the N-terminal C1q A-chain showed a stronger discrimination between SLE and controls than conventional anti-C1q.

Conclusion

In this large explorative study anti-C1q correlate with SLE overall disease activity. In contrast, peptide-antibodies are associated with specific aspects of the disease suggesting epitope-specific effects of anti-C1q in patients with SLE.

Introduction

SLE is the archetype of a systemic autoimmune disease. It is characterized by a dysregulated immune system, resulting in the generation of autoantibodies to numerous self-antigens and a broad spectrum of clinical manifestations. The exact cellular and molecular mechanisms leading to the disease remain incompletely understood[73] but may be elucidated by exploring the characteristics of self-antigens. One of these self-antigens is C1q, the first component of the classical complement activation pathway. Approximately 20–50% of unselected SLE patients have anti-C1q.[191]

Positivity for anti-C1q is predictive for flares of LN and anti-C1q levels correlate with overall disease activity.[192] Additional lines of evidence suggest that these antibodies are directly involved in tissue injury[86]: C1q deposition is a typical finding in severe LN and anti-C1q could be extracted from glomerular basement membrane fragments.[10] Furthermore, C1q is a highly functional molecule[37] and experimental data support the assumption, that binding of anti-C1q alter those functions.[16,81,97,99] However, the definite pathogenic role of the polyclonal anti-C1q remains to be determined and may strongly depend on the antibody binding site.

C1q is composed of 18 polypeptide chains (six A-, six B- and six C-chains), that form six triple helices assembling to a structure that resembles a bouquet of tulips. Each chain has a short N-terminal region, followed by an ~81 residue-long CLR forming the stalk of the molecule and a ~135 residue-long C-terminal globular head region.[33] The globular heads are mostly responsible for the recognition of target structures, e.g. Fc parts of bound Igs,[193] surface proteins of pathogens and apoptotic cells.[194] Upon binding of C1q, the CLR mediates immune effector mechanisms, including complement activation and enhancement of phagocytosis through interaction with cell surface receptors.[40,195]

Anti-C1q are polyclonal and primarily recognize neoepitopes on the CLR of C1q[24,196] and to a lower extend also on gC1q.[197,198] These epitopes are cryptic, only exposed when C1q is in its bound form[25] and certainly located in different structures. However, so far little is known about the precise C1q epitopes.[101,199] As SLE patient-derived monoclonal anti-C1q Fabs recognize

different C1q polypeptide chains in Western blot assay[101], they were used in a previous microarray-based peptide scan to identify peptide sequences recognized by anti-C1q.[98] By this approach, Vanhecke *et al.* described a major linear epitope being located on the N-terminal C1q A-chain covering the arginine rich part of the chain, the so-called "A08". Interestingly, this region is also known as a major binding site for non-Ig molecules[200], and could even be an early epitope allowing cross reactivity of antibodies that primarily target EBNA-1 of Epstein Bar Virus (EBV) due to sequence homology.[102] Epitope spreading might then lead to a more diverse antibody repertoire against the whole C1q molecule. As C1q has more functional subunits than just "A08", e.g. the globular heads, the lysins in the C-terminal CLR that mediate the interaction of C1q with the $C1s_2r_2$ tetramer[201] and widely unknown regions, which are responsible for the interaction with C1q receptors[40], antibodies targeting these structures might have different functional consequences and thus mediate different disease manifestations.

The aim of this study was to explore epitopes of C1q and determine whether epitope-specific antibodies against C1q can be linked to specific disease manifestations.

Since the study by Vanhecke *et al.*, was limited to the use of monoclonal antibodies, which do not mirror the polyclonal character of anti-C1q in patients, we used 22 SLE patient sera to determine the epitope landscape of C1q. In addition, we applied an advanced epitope mapping method based on densely overlapping linear as well as cyclized peptides, to increase sensitivity, since many epitopes rely on protein folding, which can hardly be detected with standard microarrays based on linear peptides alone.[202] Subsequently we investigated the conspicuous peptide sequences by analyzing a large cohort of well-defined SLE patients provided by the SSCS.

Hypothesis

With the large size of C1q and the polyclonal character of anti-C1q, it appears evident that some regions are potentially more immunogenic than others and have different functional consequences. Therefore, we sought to extend the previous search for novel epitopes with a high abundance using a more sophisticated approach for epitope-mapping. Furthermore, we hypothesized that epitope-specific antibodies could potentially associate with specific disease manifestations in SLE.

Methods

Blood samples

Cohort 1: For the epitope mapping, 22 serum or plasma samples were used from healthy blood donors, SLE patients being anti-C1q positive or negative respectively, and anti-C1q positive patients with diseases other than SLE. SLE patients fulfilled at least 4/11 ACR revised criteria for the classification of SLE and were all recruited at the University Hospital Basel. Sera from healthy blood donors were obtained from the blood donation center in Basel.

Cohort 2: To determine the association of epitope-specific anti-C1q with disease manifestations, serum samples and related clinical data from 378 SLE patients were provided by the SSCS. SSCS is a prospective, nationwide, multicenter and longitudinal study of SLE patients living in Switzerland.[203] SSCS includes adult SLE patients (> 17 years old) who fulfill at least 3/11 ACR revised criteria for the classification as SLE at the time of inclusion and who had given written informed consent. Patients were solely selected based on the availability of complete disease activity scores (Safety of Estrogens in Lupus Erythematosus National Assessment [SELENA]-SLEDAI and Physician's Global Assessment [PGA]) and available plasma sample at the time of study visit.

Plasma samples of 100 healthy, sex-matched blood donors from the blood donation center in Basel served as a reference.

Data collection

Samples and data from SLE patients and healthy blood donors were collected cross-sectionally between October 2010 and June 2018. Laboratory parameters were assessed by the individual centers. SLE manifestations were defined using the ACR revised classification criteria.[154,155] Disease activity was assessed by the SLEDAI score with the SELENA modification.[204]

Additionally, we used a PGA score with a 4-point scale of disease activity, ranging from 0 (inactive) to 3 (very active). Both scores were used with a 30-day window.[205] Active SLE disease was defined as a SELENA-SLEDAI ≥ 6 and PGA ≥ 1 at the time of sampling.

Epitope mapping

Peptide microarrays were manufactured by PEPperPRINT (Heidelberg, Germany). The peptide sequence of the CLR of C1q was laser printed in an array format. Measurements were performed

with cyclized peptides of 7, 10, and 13 AA length and with linear peptides of 15 AA length. The cyclic constrained peptides were linked at the C- and N-terminus by a thioether linkage and anchored to the microarray surface. The linear peptides were printed as stripes continually bound to the surface of the microarray. Both linear and conformational cyclic peptides were expressed with a 1-AA shift. Peptide microarrays were screened according to the manufacturer's protocol[206] with the following specifications: The secondary antibody was a goat anti-human IgG (Fc) DyLight680 and a mouse monoclonal anti-HA (12CA5) DyLight800 antibody was used as a control. Assays were performed with serum or plasma dilutions of 1:500. Arrays were scanned using a LI-COR Odyssey Imaging System and microarray image analyses were done with PepSlide® analyzer. The optical density (OD) was converted to a digital scale leading to values that ranged from 0 to 16,052 arbitrary fluorescence units (aFU) in our study. The magnitude of binding intensity of IgG to certain peptides is presented as color in a Heatmap, in which the highest value was limited to 1,000 aFU to facilitate comparisons between binding intensities of smaller amplitude.

C1q-derived peptides

Peptides used for peptide ELISA were synthesized with ≥ 95% purity by peptides & elephants GmbH (Hennigsdorf, Germany) and named according to the position of their first AA in the C1q molecule.[33] Accordingly, the previously described "A08" was renamed A15 in this study.[98] The difference in numbering is due to the two AA increments used previously, while the current study used one AA increment being identical with the AA position in the molecule. Peptides used for the experiments are summarized in Table 4. The peptides were diluted in Invitrogen™ UltraPuren™ DNase/RNase-Free distilled water and stored at -80°C until further use.

Table 4 | Nomenclature and structure of the studied peptides

Previous name	New Name	C1q chain	N-term	Sequence
	A09	A	Biotin	GKKGEAGRPGRRGRP
A08	A15	A	Biotin	GRPGRRGRPGLKG
	A86	A	Biotin	NIKDQPRPAFSAIRR
	B41	B	n/a	cyclo[K(biotin)AGDHGEF]
	B43	B	n/a	cyclo[K(biotin)DHGEFGE]
	B83	B	n/a	cyclo[K[biotin)GESGDY]

A15 and A09 both contain the "A08" core sequence (marked gray) described previously.[102] The B-chain-derived peptides are brought into a cyclic conformation by an amide bond.

Peptide and anti-C1q ELISAs

Peptide and anti-C1q ELISAs were performed as published previously[98], with some modifications to improve the signal to noise ratio. The ELISAs were performed throughout with TBS and the peptide ELISAs were incubated at 33°C instead of 27°C. The incubation step of peptide coating was shortened to one hour. The optimal serum dilutions were found to be 1:50 for the anti-C1q ELISA, as well as for the peptide ELISAs of B-chain-derived peptides. The optimal serum dilution for ELISAs of A-chain-derived peptides was 1:100. Before diluting the samples to their final concentration, they were vortexed and then centrifuged for 30 min at 4°C and 14,000 *g*.

Bound antibodies to peptides or C1q were detected by incubation for 45 min (peptides) or one hour (C1q) with AP-conjugated goat anti-human Fc(gamma) antibody diluted 1:1,000 (peptide ELISAs) or 1:5,000 (anti-C1q ELISAs). For the peptide ELISAs, the signal obtained from incubating every single sample with diluting buffer instead of peptide was considered background, and this OD value was subtracted from the peptide-specific peptide.

For further analyses we standardized the experiments by expressing the data in units relative to the OD values obtained from a reference SLE serum (set as 1,000 relative Units, reIU), which was used to establish a standard curve. The reference serum showed high level of binding in the peptide ELISA and anti-C1q ELISA respectively and was included on every second plate. Calibration curves were fitted using a sigmoidal four-parameter logistic model. If the background of a peptide ELISA was higher than foreground, reIU were set to zero. If the background-adjusted

values were higher than the upper limit of the standard curve, measurements were repeated in a 1:1,000 dilution and if necessary, in a 1:10,000 dilution, for the A-chain-derived peptides and in a 1:500, and if necessary 1:5,000 dilution, for the B-chain-derived peptides. For each serum, all peptide ELISAs were performed simultaneously.

Statistical analysis

Statistical analyses and graphical presentations were conducted using R software version 4.0.2. and GraphPad Prism version 9.1.0. Univariate analyses were used to describe baseline characteristics. Data for continuous variables are presented as median with interquartile range (IQR). Categorical data are presented as frequency and percentage.

Non parametric-tests were used throughout, because of a lack of normal distribution in peptide- and anti-C1q ELISA. Correlations were analyzed by Spearman's correlation coefficient and differences in antibody titers were analyzed by a two-sided Mann-Whitney test. Statistical significance was considered as $*P \leq 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ respectively. For the ELISA data, we set a cutoff corresponding to < 10% positivity of the controls in all assays. Univariate logistic regression models were used to examine the relationship between positivity in ELISAs and manifestations of SLE, taking the serological measures as predictors and the presence of different disease features as dependent variables. In addition, we examined the association between positivity in peptide ELISAs and disease duration at the time of blood sampling, taking disease activity as a potential confounder into account. Since we performed an explorative study with no prespecified key hypothesis, type I error control was not implemented. Statistical tests are therefore used only for descriptive purposes. We expressed the results of the logistic regression analyses as odds ratios (OR) with associated 95% confidence intervals (95% CI). Intervals have not been adjusted for multiplicity. Subsequently we performed receiver operating characteristic (ROC) curves to compare the diagnostic performance of the peptide- and anti-C1q ELISAs with regard to specific outcomes. To compare the AUC of two ROC curves, DeLong test was used.

Compliance with ethical standards

This study was approved by all responsible local ethical committees and Swissethics (Ethical Committee of the Canton Vaud, Switzerland Ref. No. 2017-01434). All procedures performed in this study involving human participants were in accordance with the ethical standards of the research committee and with the 1964 Helsinki declaration and its later amendments or

comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Results

Patients characteristics

Cohort 1: Demographic and baseline characteristics of patients used for epitope mapping are summarized in Supplementary table 1 Part III (page 91). Patient SLE 4 had been described in a previous case report.[156] Patients with anti-C1q but disease other than SLE had complement C2 deficiency (n = 2)[207], HUVS (n = 1)[208] and essential cryoglobulinemia (n = 1)[209].

Cohort 2: A total of 378 patients, of previously selected 392 SLE patients, met the inclusion criteria. The flow diagram of eligible patients is shown in Supplementary figure Part III (page 92). Demographic and clinical characteristics are summarized in Table 5.

Of the 378 patients 324 (85.7%) were female and 54 (14.3%) were male. The median age at the time of blood sampling was 42 (32–54) years and the median SLE disease duration since diagnosis was 5 (1–13) years. At the time of the study visit 131/378 (34.7%) of the patients had active disease, defined as a PGA ≥1 and SELENA-SLEDAI ≥6.[204] The main clinical manifestations of the study population as defined by the SELENA-SLEDAI are shown in Table 5. The sex-matched control group consisted of 85 (85%) women and 15 (15%) men. Their median age at the time of blood sampling was 48 (38–60) years.

Table 5 | Demographic and clinical characteristics of patients with SLE and control group (normal blood donor)

	SLE group, *n* = 378†	Control group, *n* = 100
Female, *n* (%)	324 (85.7)	85 (85)
Male, *n* (%)	54 (14.3)	15 (15)
Disease classification at time of inclusion		
ACR, median (IQR)	5 (4–6)	
Ethnicity		
Caucasian, *n* (%)	280 (74.1)	
African, *n* (%)	38 (10.1)	
Asian, *n* (%)	37 (9.8)	
Native American, *n* (%)	18 (4.8)	
Other, *n* (%)	2 (0.5)	
Unknown, *n* (%)	3 (0.8)	
Age		
At blood sampling, median (IQR)	42 (32–54)	48 (38–60)
Disease duration since diagnosis of SLE (IQR)	5 (1–13)	
Disease activity and clinical features		
Active disease‡, *n* (%)	131 (34.7)	
Fever, *n* (%)	24/377 (6.4)	
Arthritis, *n* (%)	84/375 (22.4)	
Active muco-cutaneous involvement§, *n* (%)	119/373 (31.9)	
Vasculitis, *n* (%)	8/377 (2.1)	
Serositis, *n* (%)	22/372 (5.9)	
CNS involvement¶, *n* (%)	12/375 (3.2)	
Leukopenia, *n* (%)	53/372 (14.2)	
Thrombocytopenia, *n* (%)	31/373 (8.3)	
Proteinuria, *n* (%)	56/298 (18.8)	
Hematuria, *n* (%)	63/340 (18.5)	
Low Complement, *n* (%)	112/341 (32.8)	
Anti-dsDNA antibodies, *n* (%)	167/340 (49.1)	
Anemia, *n* (%)	126/371(34.0)	
Elevated ESR#, *n* (%)	103/339 (30.4)	
Anti-Phospholipid antibodies, *n* (%)	59/183 (32.2)	

†n = 378 unless otherwise stated, ‡active disease was defined as SELENA- SLEDAI ≥ 6 and PGA ≥ 1, §Active muco-cutaneous involvement defined as malar rash or mucosal ulcers or alopecia at time point of blood sampling, ¶CNS involvement was defined as psychosis, seizure or organic brain syndrome at time of blood sampling, #ESR = erythrocyte sedimentation rate.

Epitope mapping

In a first step we investigated the binding of IgG to peptides covering the CLR of the C1q A-, B- and C-chain, using peptides in different lengths and in a linear as well in a cyclic conformation. We initially tested eight samples; four from anti-C1q positive SLE patients, two from anti-C1q negative SLE patients and two from healthy control donors.

Results are shown in Figure 19. Signal intensities from 10 and 13 AA cyclic peptides yielded similar results independent of the peptide lengths and were less strong than from 7 AA peptides. Considering the 7 AA cyclic peptides, a peak-signal was observed in peptides 13 to 19 of the A-chain, all containing the previously described "A08" core sequence.[102] The cyclic B-chain-derived peptides showed two peaks at position 41 and 43, which were not present in the linear conformation. In the following these peptides are called B41 and B43. The C-chain-derived peptides did not show consistent signal elevations. Within the 15 AA peptides in linear conformation, two constant signals appeared at position 9 and 86 of the C1q A-chain, hereafter referred to as A09 and A86 respectively. With regard to the B- and C-chains, we did not observe patterns of binding intensities shared by several SLE patients to any of the 15-AA peptides in linear conformation.

In a second step we analyzed 16 additional serum samples, covering a broader disease spectrum, but limited the analysis to 7 AA cyclic peptides from the whole CLR of the C1q B-chain and the N-terminal part of the C1q A-chain covering the already described "A08" epitope. Among all samples, two sera were investigated in both experiments and served as an internal control. Results are shown in Supplementary figure 2 Part III (page 92). Binding intensities to peptides B41 and B43 were detected in all patient groups, but were slightly lower in healthy donors. Similarly, binding to peptide position 83 of the B-chain (B83) can be seen in all patient groups, although less pronounced in the control group.

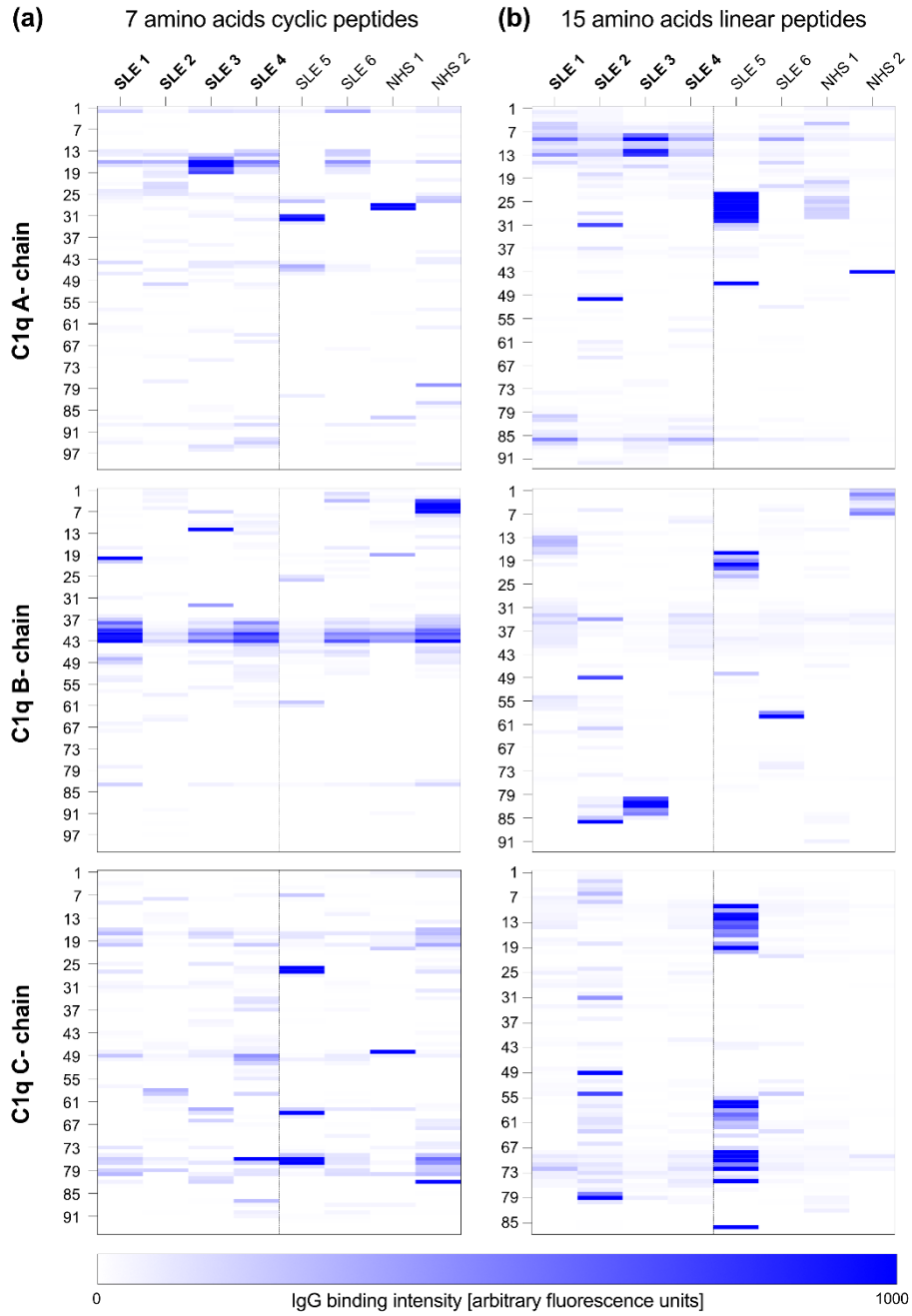


Figure 19 | Epitope mapping of the CLR of C1q. Six patients with SLE and two healthy blood donors were screened for antibodies against peptides of the CLR of C1q (A-, B- and C-chain)

The heatmap color represents the intensity of the antibody binding signal in each sample (column) to each peptide, named according to the position of their first AA on the C1q molecule (rows, left site). Patients in bold were anti-C1q positive at the time of blood collection, all others anti-C1q negative. **(a)** 7 AA peptides in cyclic confirmation. **(b)** 15 AA linear peptides.

Prevalence and clinical association of autoantibodies

To characterize the clinical significance of the candidate epitopes which stand out in the epitope mapping, we established a peptide ELISA. Examined peptides are shown in Table 1. As an internal control, we measured antibodies against A15 (anti-A15; formerly called 'anti-A08') and anti-C1q as well. Figure 20 shows the distribution of measured autoantibodies in SLE patients and controls as well as their correlation among each other. SLE IgG showed significantly higher binding to C1q, A15 and A09 and slightly lower binding to B41 when compared to controls. No significant differences in IgG binding were observed for the other peptides.

Since anti-C1q autoantibodies are present in up to 10% of healthy individuals and in analogy to previous study data[90,187], we chose a cutoff for positivity by accepting < 10% positive healthy blood donors in all assays. With this cutoff, 65/378 (17%) of the SLE patients were anti-C1q positive, 159/378 (42%) anti-A09 positive, 123/378 (32.5%) anti-A15 positive, 34/378 (8.9%) anti-A86 positive, 32/378 (8.5%) anti-B41 positive, 53/378 (14%) anti-B43 positive and 29/378 (7.7%) anti-B83 positive. Antibodies directed against C1q-derived epitopes correlated weakly with antibodies against intact C1q (anti-C1q) (r = 0.1–0.2). Anti-A09 and anti-A15 showed a strong correlation with each other (r = 0.7), but both had only a moderate correlation with anti-A86 (r = 0.4). Autoantibodies directed against B-chain-derived epitopes showed strong correlations among each other (r = 0.6) but only weak correlations to A-chain-derived epitopes (r = 0.1 – 0.3).

To explore the assumed relationship between measured autoantibodies and manifestations of SLE, univariate logistic regression was conducted, taking positivity in ELISAs as binary predictor and the presence of different disease features as binary dependent variable. Figure 21 shows OR's and 95% CI's of SLE features as a function of positivity in anti-C1q- and A-chain-derived peptide ELISAs. The corresponding values in numbers are shown in Supplementary table 2 Part III (page 94).

While anti-C1q positivity correlated strongly with overall disease activity as well as with several SLE features, positivity for autoantibodies to A-chain-derived peptides correlated only weakly with disease manifestations: Anti-A09 correlated with fever, arthritis, thrombocytopenia, and with the occurrence of anti-dsDNA antibodies. Patients with anti-A15 had an increased probability of having active disease, arthritis, leukopenia, low complement and antiphospholipid antibodies. Patients with anti-A86 were more likely to have vasculitis and leucopenia. Univariate logistic regression for anti-B-chain-derived peptide-ELISAs showed solely a weak correlation between anti-B43 and arthritis (OR = 1.995, CI = 1.046–3.708) and anti-dsDNA antibodies (OR

= 2.387, CI = 1.232–4.824) and are shown in Supplementary figure 3 Part III (page 93) and Supplementary table 3 Part III (page 94).

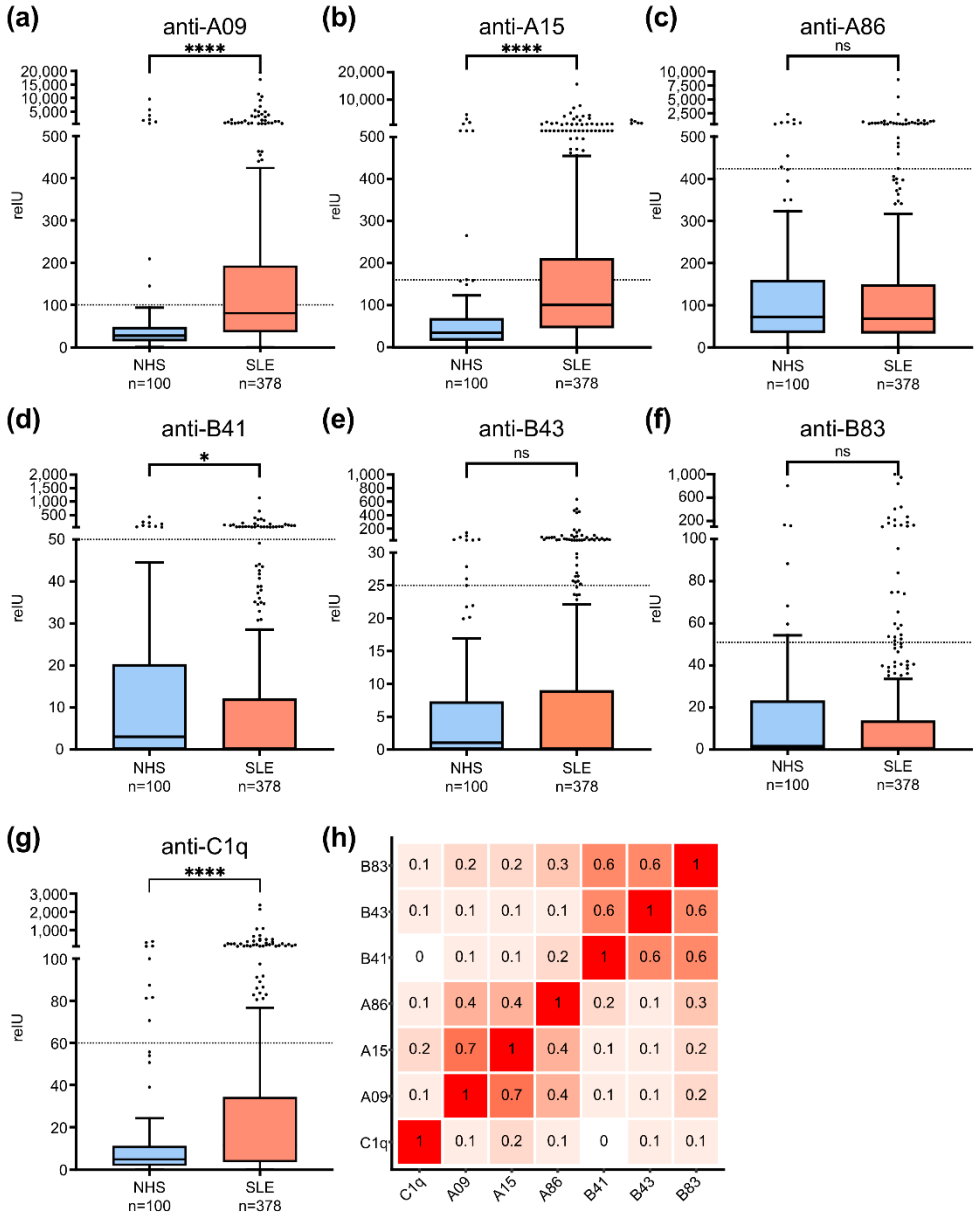


Figure 20 | Binding of IgG from SLE patients and healthy controls to candidate epitopes of the CLR and correlation of autoantibodies among each other

(a–g) Graphs are named according to examined epitopes and show Tukey's boxplots with whisker lengths of 1.5x interquartile range. Outliers are shown as dots. Since the data are markedly skewed, Y-axis is segmented. Cutoffs for positivity are indicated by dashed lines. Statistical significance was considered as $*P \leq 0.05$, $****P < 0.0001$ respectively, ns, not significant. **(h)** Correlation-plot showing spearman correlation coefficients of measured autoantibodies among each other.

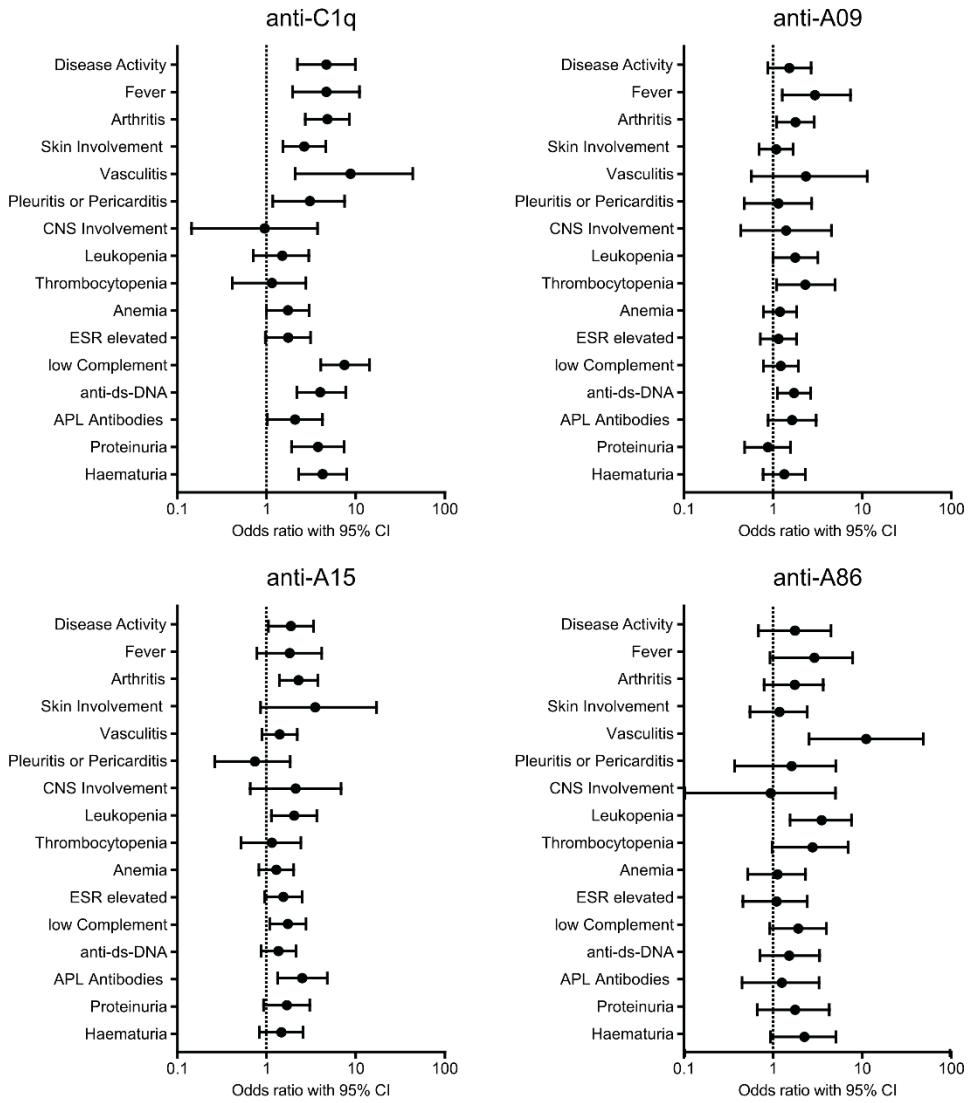


Figure 21 | Univariate logistic regression

Positivity in ELISAs as binary predictor and presence of disease manifestations as binary dependent variable. The graphs show odds-ratios and 95% confidence intervals of SLE manifestations. ESR, erythrocyte sedimentation rate; APL, antiphospholipid.

Since it was previously described that anti-A15 (formerly called 'anti-A08') show a stronger correlation with SLE disease activity and nephritis than anti-C1q[99], we established additional ROC curves to allow a better interpretation of the diagnostic performance regarding those endpoints. ROC curves are shown in Figure 22. Regarding the discrimination between SLE patients and healthy donors, autoantibodies directed against the N-terminal part of the A-chain had a significantly larger AUC than anti-C1q (0.75 versus 0.64; $P < 0.01$). In contrast, the diagnostic performance of those autoantibodies taking the outcome active disease into account was not significantly different (AUC anti-A09: 0.59, AUC anti-A15: 0.61, AUC anti-C1q: 0.66; $P = 0.09$ for comparison of anti-A09 vs anti-C1q and $P = 0.19$ for anti-A15 vs anti-C1q).

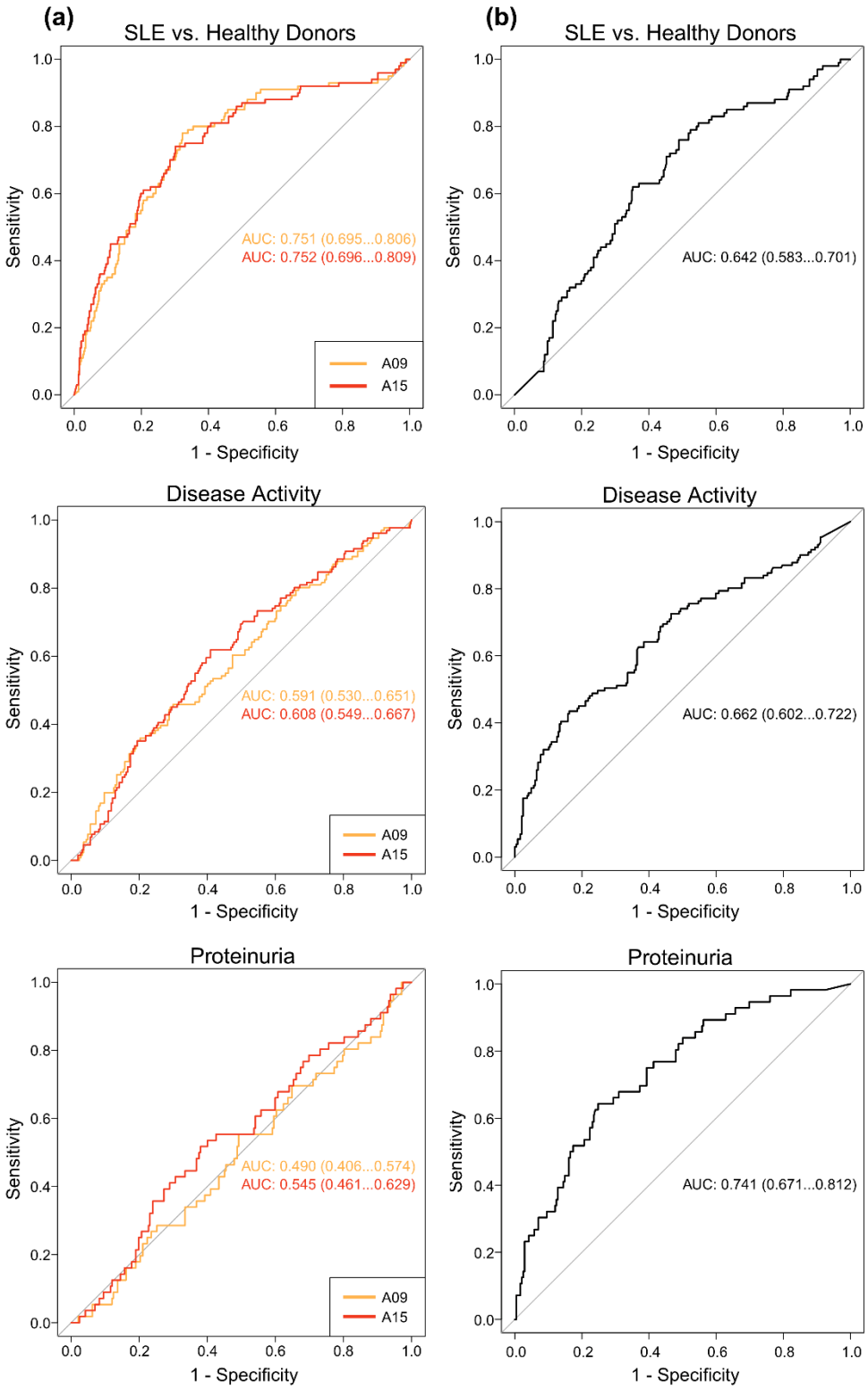


Figure 22 | Comparison of the diagnostic performance between anti-C1q and anti-A09/A15 as determined by ELISA

ROC curves analyzing the diagnostic performance of anti-A09, anti-A15 and anti-C1q regarding the discrimination of SLE patients from healthy donors, SLE patients with active versus inactive disease and proteinuria versus no proteinuria. **(a)** ROC curves of anti-A09 and anti-A15. **(b)** ROC curves of anti-C1q.

Regarding the occurrence of proteinuria, which is a typical finding in LN, anti-C1q show significantly better diagnostic performance than anti-A09 and anti-A15 respectively (AUC anti-A09: 0.49, AUC anti-A15: 0.55, AUC anti-C1q: 0.74; $P < 0.0001$ comparing anti-C1q versus anti-A09 and anti-A15 respectively).

To investigate the association between anti-C1q, -A09 and -A15 and disease duration we conducted a multivariate logistic regression, adjusting for disease activity. Supplementary figure 4 Part III (page 95) shows the graphical presentation of this multivariate regression using disease duration and activity as predictors and the possibility of positive autoantibodies as outcome. Adjusted Odds ratio for being autoantibody positive per one year of disease duration was 0.94 (CI's = 0.9–0.98) for anti-C1q, 0.98 (CI's = 0.95–1) for anti-A09 and 0.97 (CI's = 0.95–0.99) for anti-A15. The probability of having active disease was markedly higher when having positive anti-C1q (OR_{adj} = 4.86, CI's = 2.67–9.08), than anti-A09 (OR_{adj} = 1.43, CI's = 0.9–2.27) or anti-A15 antibodies (OR_{adj} = 1.67, CI's = 1.03–2.68). There was no evidence of multicollinearity.

Discussion

This study aimed at identifying and exploring the clinical relevance of epitope-specific autoantibodies against complement C1q (anti-C1q) in patients with SLE. Considering the multiple functions of C1q, the role of C1q in SLE as well as the striking association of anti-C1q with active LN and SLE disease activity, a better understanding of epitopes of C1q could aid understanding the pathogenic mechanisms of SLE as well as improving diagnostic procedures. By using SLE patient sera in an advanced epitope mapping method, we first identified three epitopes on the C1q A- and three on the B-chain. In subsequent exploration of clinical relevance of these epitopes in a large cohort of patients by peptide-ELISA, two of the investigated peptides were significantly better recognized by serum IgG of SLE patients than of healthy controls. In addition, positivity for four of the investigated peptide-specific antibodies showed associations with selected SLE disease manifestations. These primarily explorative analyses might point to distinct functional properties of the measured peptide-specific antibodies.

The most obvious association with SLE was found for IgG antibodies targeting an epitope on the N-terminal C1q A-chain. The corresponding peptides were named 'A09' and 'A15' respectively, based on the position of their first AA on the CLR of C1q. Anti-A09 correlated with fever, arthritis, thrombocytopenia, and the occurrence of anti-dsDNA antibodies, while patients with anti-A15 had an increased probability of having active disease, arthritis, leukopenia, low complement and antiphospholipid antibodies. Regarding the occurrence of proteinuria, which is a typical finding in LN, anti-C1q showed significantly better diagnostic performance than anti-A09 as well as anti-A15. This finding apparently is not in line with previous studies which showed that anti-A15-ELISA is more specific and more sensitive than a conventional anti-C1q assay for the detection of active LN in SLE patients.[98,99] These differences in observation might be explained by the differences in patient selection and number. Both previous studies examined exclusively[99] or predominantly[98] lupus patients with renal biopsy-proven LN, whereas most patients analyzed in the present study had long lasting, stable disease resembling an unselected clinical outpatient cohort of patients with closely monitored disease. Furthermore the sample sizes of the preceding studies were substantially smaller, than in the present study, namely $n = 61$ (Ref 98) and $n = 210$ (Ref 99) versus $n = 378$ in our study presented here.

Nevertheless the present study is in line with the study from Vanhecke *et al.* showing that anti-A15 is better in discriminating asymptomatic donor sera from SLE patient sera than anti-C1q. In the present study, this discrimination was in the same range as the reported diagnostic

performance of anti-dsDNA antibodies.[210] Hence, anti-A-15 might serve as a diagnostic marker for SLE. However, to determine the real discriminatory power, it will be of importance to also investigate anti-A09 and -A15 in other inflammatory rheumatic diseases, and to perform a direct comparison with anti-dsDNA antibodies.

Furthermore, anti-C1q showed a weak correlation with anti-A09 or anti-A15 antibodies, respectively as also observed by Vanhecke *et al.*[98] In line with these findings, Wu *et al.* recently described that anti-A15 antibodies derived from 10 LN patients bound to A15 but not to intact C1q.[211] Regarding the potential functional consequences of anti-A09 and -A15, it should be noted that both peptides include a major binding site of C1q for non-Ig molecules.[200] With regard to the interaction of A09 compared to A15 with binding partners other than anti-C1q, we hypothesized that the arginines being present in A09 as well will lead to very similar interactions as observed for A15. However, the differences in correlation with clinical parameters between anti-A09 and ant-A15 suggest that either the antibodies have a different potential to interfere with the known interactions of C1q with the described non-immunological molecules and/or receptors, or may point to differences in interaction between the two sites themselves with these binding partners.

The mentioned core sequence was previously described to allow cross-reactivity between antibodies directed against EBNA-1 of EBV and C1q.[102] In addition, Wu *et al.* could show that BALB/C mice, which were immunized with the A15 peptide, developed anti-C1q antibodies. They concluded that A15 is important for development of anti- C1q antibodies, but epitope spreading might then lead to a more diverse antibody repertoire against the whole C1q molecule. In line with this finding, generation of anti-A09 and -A15 in SLE patients seem to be an early event in the course of the disease. Additionally, data from multivariate regression suggest that anti-A09 and -A15 have higher stability over time with lower dependency on disease activity (Supplementary figure 4 Part III, page 95). Taken together, these results support the hypothesis, that molecular mimicry is an early event in the pathogenesis of SLE, with the formation of anti-A15 antibodies being an intermediate step but might also explain the weak correlation between anti-A15 and anti-C1q.

With regard to IgG antibodies against the other described peptide epitopes, no overall differences in antibody levels between SLE patients and control sera were observed. However, when judging on the significance of these antibodies, one has to keep in mind that quantitatively peptide-specific anti-C1q are only representing a small fraction of total anti-C1q, and they only occur in a subgroup of patients that is likely to be too small to have an impact on overall differences between unselected SLE patients and healthy controls. As the study hypothesis was that antibodies

against distinct epitopes of the multifunctional C1q molecule are associated with a specific disease expression, we thus also explored the association of the peptide antibody positivity with the clinical presentation of SLE. Patients with anti-A86 were more likely to have vasculitis and leucopenia, and the presence of anti-B43 was associated with arthritis and anti-dsDNA antibodies.

However, because of the explorative character of these analyses, confirmatory studies in large cohorts of SLE patients covering a broad spectrum of SLE manifestations and taking interrelations into account will be required to define the definite role of the described peptide antibodies.

Lastly, with regard to anti-C1q levels we observed that anti-C1q are associated with a much wider range of clinical disease manifestations than previously described. So far, anti-C1q antibodies have mainly been studied in association with LN.[212–214] However, while confirming this known association in the present study, we also observed a clear association with arthritis OR = 4.811 (2.722–8.543), skin involvement OR = 2.646 (1.522–4.613), vasculitis OR = 8.757 (2.093–43.629) and serositis OR = 3.065 (1.175–7.514). Therefore, anti-C1q could be more broadly considered as marker of SLE disease activity. This observation could be due to the large number of SLE patients investigated in our study. To the best of our knowledge, to date our study is the largest ever on anti-C1q in SLE patients.

The main limitation of the present study is its retrospective observational character. In addition, in spite of the large number of investigated patients, the sample size was still too small to make a clear statistical statement for some of the investigated disease features and thus would require even larger cohorts. Moreover, longitudinal data on the described antibodies will be of importance in the future. Lastly, despite the extensive character of our epitope mapping, the expression of peptides only partially resembles the conformation of the corresponding peptide sequences as part of the complete C1q molecule, and the expression of peptides probably differed between their expression in the initial epitope mapping versus the ELISAs performed in the large SLE cohort. Furthermore, our methodologies were not able to detect and describe antibodies against epitopes involving two or more chains of C1q. Thus, the described peptide-specific antibodies are likely still representing only a fraction of total anti-C1q.

In conclusion, in this exploratory and largest study to date on anti-C1q in SLE patients we describe six candidate epitopes of anti-C1q and their clinical associations in SLE patients. Two N-terminal located A-chain epitopes, which provide good discrimination between SLE patients and healthy individuals, might serve as a biomarker of the disease. In addition, peptide-specific anti-C1q were found to be associated with specific disease manifestations, but their potential

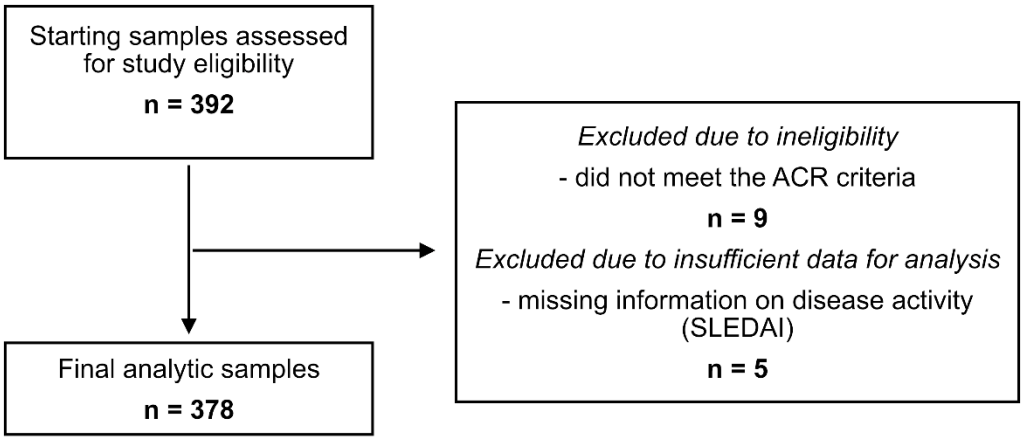
impact on clinical patient management and for the understanding of pathogenic mechanisms needs to be confirmed.

Supplementary Information Part III

Supplementary table 1 Part III | Baseline demographics and disease characteristics in patients used for epitope mapping

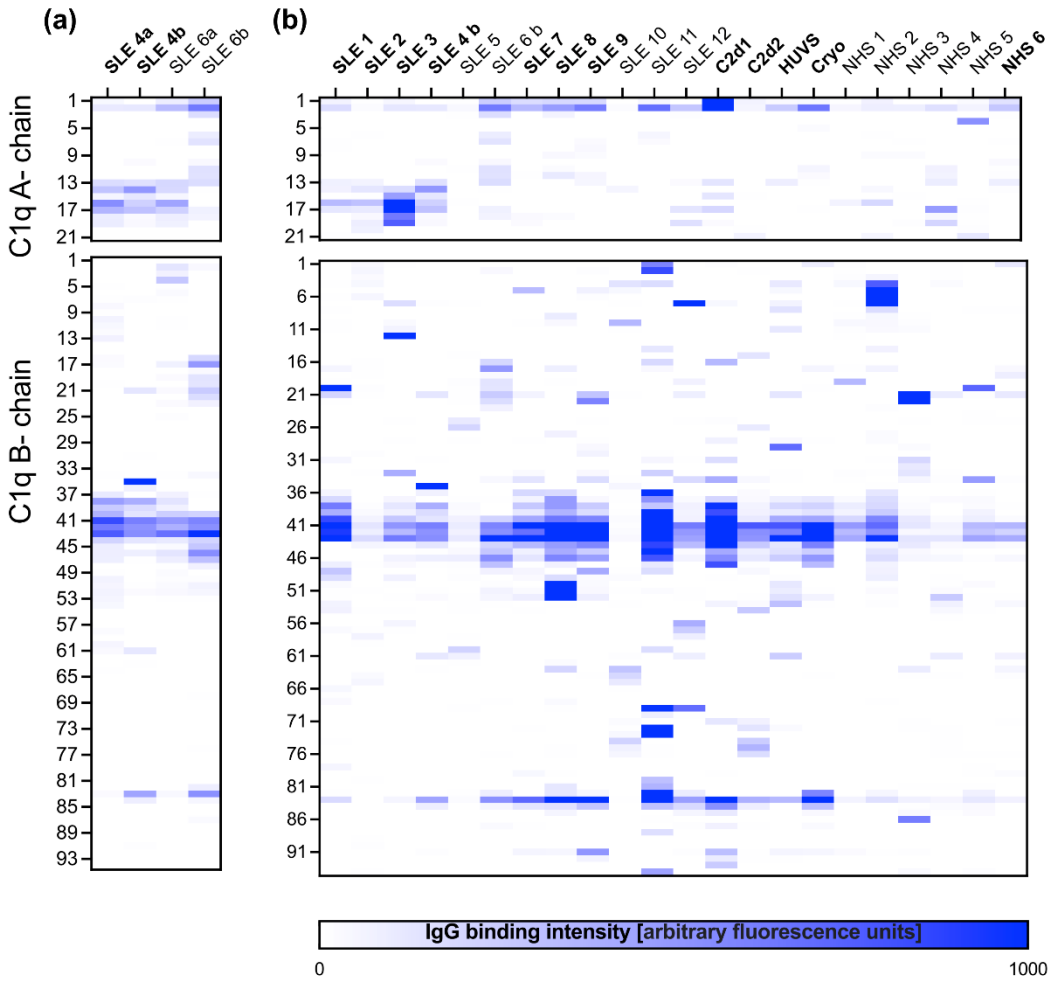
Sample	Dx.	Anti-C1q IgG	Clinical state	SLEDAI score	Age [y]	sex	Class of Lupus Nephritis (WHO)	Disease-Duration [y]
SLE 1	SLE	pos.	active	16	54	m	IV	0
SLE 2	SLE	pos.	active	17	52	f	IV	1.5
SLE 3	SLE	pos	active	10	24	f	III	0
SLE 4	SLE	pos.	active	10	32	f	IV	0
SLE 5	SLE	neg.	inactive	0	76	m	III	11
SLE 6	SLE	neg.	active	42	70	f	-	0
SLE 7	SLE	pos.	moderate	6	52	f	-	21
SLE 8	SLE	pos.	active	24	44	f	IV	4
SLE 9	SLE	pos.	active	38	49	m	IV	12
SLE 10	SLE	neg	inactive	6	65	f	III	13
SLE 11	SLE	neg.	inactive	3	62	f	IV	26
SLE 12	SLE	neg.	inactive	5	50	f	V	18
C2d1	other	pos.	active	-	57	m	-	9
C2d2	other	pos.	inactive	-	58	f	-	-
HUVS	other	pos.	active	-	59	f	IIa, Vb	unk.
Cryo	other	pos.	active	-	52	m	secondary MPGN type I	4
NHS 1	-	neg.	-	-	31	f	-	-
NHS 2	-	neg.	-	-	33	f	-	-
NHS 3	-	neg.	-	-	34	m	-	-
NHS 4	-	neg	-	-	unk.	unk.	-	-
NHS 5	-	neg.	-	-	unk.	unk.	-	-
NHS 6	-	pos.	-	-	28	f	-	-

Characteristics of Patients included in the epitope-mapping. The clinical state was classified according to physician's global assessment index at the time of study visit. Anti-C1q-status was determined in the University Hospital Basel and by non-commercial anti-C1q ELISA. SLE = Systemic Lupus Erythematosus, NHS = Normal Human Serum, C2d1 and C2d2 = patients with complement C2 deficiency, HUVS = Hypocomplementic Urticaria Vasculitis Syndrome, Cryo = Patient with severe primary cryoglobulinemia, Dx= Diagnosis, SLEDAI = Systemic Lupus Erythematosus Disease Activity Index, ACR = American College of Rheumatology * assessment at time of sampling.



Supplementary figure 1 Part III | Flow chart of included and excluded patients

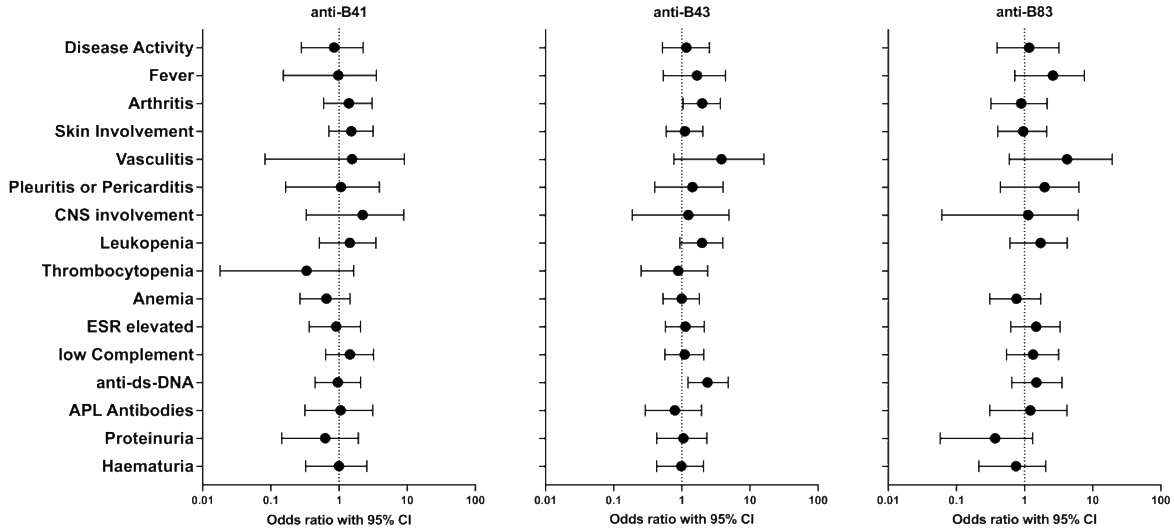
ACR = American College of Rheumatology. SLEDAI = System lupus erythematosus disease activity index.



Supplementary figure 2 Part III - Epitope mapping of the CLR of C1q

Among the 16 samples used for the second epitope mapping, 2 were investigated in both experiments and served as controls (SLE 4 and 6). The appendix **(a)** refers to results obtained in experiment 1 and **(b)** for results obtained in

experiment 2. Experiment 2 was limited to 7 amino acid cyclic peptides, derived from the whole CLR of the C1q B-chain and the N-terminal part of the C1q A-chain covering the "A08" epitope (renamed A15). The heatmap color represents the intensity of the antibody binding in each sample (column) to each peptide, named according to the position of their first amino acid on the C1q molecule (rows, left). Patients in bold were anti-C1q positive at the time of blood collection, all others anti-C1q negative. **(a)** results of the binding intensities to peptides comparing binding intensities measured in the first versus the second experiment. **(b)** Pooled analyses of IgG binding to peptides in different patient groups.



Supplementary figure 3 Part III | Univariate logistic regression with positivity in ELISAs as binary predictor and presence of disease features as binary dependent variable

The graphs show odds-ratios and 95% confidence intervals of SLE features. ESR = erythrocyte sedimentation rate. APL = antiphospholipid-antibodies. Odds ratio for thrombocytopenia in anti-B83 positive patients could not be calculated, since no anti-B83 positive patient had thrombocytopenia.

Supplementary table 2 Part III | ORs and corresponding 95% CI's resulting from univariate logistic regression, using anti-C1q, anti-A09, anti-A15 and anti-A86 as predictor

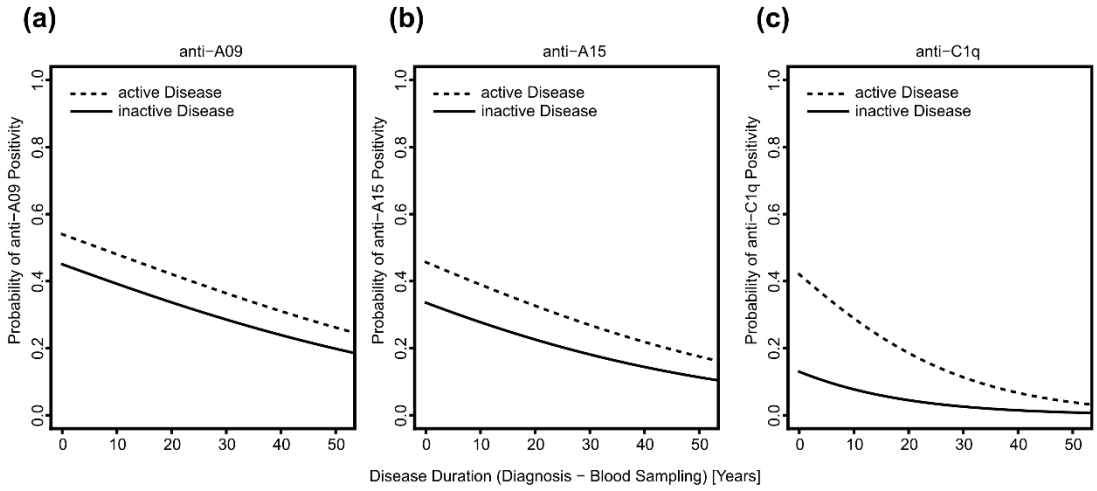
Disease Feature	anti-C1q	anti-A09	anti-A15	anti-A86
Disease Activity	4.596 (2.22–9.889)	1.528 (0.871–2.688)	1.905 (1.06–3.427)	1.77 (0.683–4.525)
Fever	4.685 (1.963–1.026)	2.972 (1.272–7.501)	1.845 (0.787–4.253)	2.94 (0.922–7.95)
Arthritis	4.811 (2.722–8.543)	1.791 (1.1–2.931)	2.317 (1.406–3.82)	1.756 (0.791–3.695)
Skin Involvement	2.646 (1.522 -4.613)	1.084 (0.697–1.681)	1.424 (0.899–2.248)	1.183 (0.548–2.44)
Vasculitis	8.757 (2.093 -43.629)	2.353 (0.569–11.609)	3.95 (0.866–17.738)	11.3 (2.559 – 50.014)
Pleuritis or Pericarditis	3.065 (1.175–7.514)	1.151 (0.474–2.738)	0.747 (0.262–1.869)	1.625 (0.367–5.112)
CNS involvement	0.952 (0.144–3.725)	1.404 (0.432–4.568)	2.157 (0.661–7.032)	0.94 (0.051–5.073)
Leukopenia	1.503 (0.714–2.979)	1.78 (0.993–3.217)	2.074 (1.145–3.745)	3.536 (1.554–7.69)
Thrombocytopenia	1.151 (0.413–2.762)	2.312 (1.1 -5.044)	1.16 (0.52–2.464)	2.8 (0.974–7.05)
Anemia	1.736 (1.002–2.991)	1.198 (0.776–1.848)	1.303 (0.826–2.048)	1.123 (0.519–2.33)
ESR† elevated	1.745 (0.968–3.108)	1.152 (0.719 -1.838)	1.568 (0.964–2.541)	1.094 (0.456–2.445)
low Complement	7.482 (4.072–14.297)	1.222 (0.775–1.927)	1.759 (1.094–2.824)	1.928 (0.916–4.028)
anti-dsDNA	4.004 (2.181–7.733)	1.724 (1.12–2.664)	1.377 (0.875–2.172)	1.521 (0.708–3.364)
APL-Antibodies	2.086 (1.021–4.246)	1.639 (0.879–3.073)	2.559 (1.345–4.906)	1.256 (0.445–3.312)
Proteinuria	3.772 (1.904–7.4)	0.873 (0.477- 1.572)	1.713 (0.932–3.112)	1.778 (0.661–4.328)
Hematuria	4.255 (2.28–7.911)	1.339 (0.771–2.322)	1.486 (0.837–2.606)	2.263 (0.931–5.152)

ESR = erythrocyte sedimentation rate. APL = antiphospholipid. Odds ratio for thrombocytopenia in anti-B83 positive patients could not be calculated since no anti-B83 positive patient had thrombocytopenia.

Supplementary table 3 Part III | ORs and corresponding CI's resulting from univariate logistic regression, using anti-B41, anti-B43 and anti-B83 as predictor

Disease Feature	anti-B41	anti-B43	anti-B83
Disease Activity	0.845 (0.281–2.267)	1.168 (0.517–2.544)	1.166 (0.395–3.2)
Fever	0.979 (0.152–3.549)	1.672 (0.535–4.387)	2.624 (0.722–7.602)
Arthritis	1.398 (0.591–3.057)	1.995 (1.046–3.708)	0.896 (0.322–2.15)
Skin Involvement	1.517 (0.708–3.161)	1.115 (0.592–2.041)	0.957 (0.403–2.113)
Vasculitis	1.558 (0.082–9.159)	3.828 (0.766–16.094)	4.222 (0.599–19.365)
Pleuritis or Pericarditis	1.067 (0.165–3.901)	1.433 (0.401–4.042)	1.968 (0.442–6.267)
CNS involvement	2.22 (0.331–8.924)	1.252 (0.189–4.928)	1.131 (0.061–6.154)
Leukopenia	1.439 (0.514–3.474)	1.985 (0.932–3.996)	1.723 (0.609–4.237)
Thrombocytopenia	0.334 (0.018–1.648)	0.886 (0.254–2.389)	
Anemia	0.654 (0.267–1.452)	1 (0.532–1.827)	0.763 (0.308–1.724)
ESR elevated	0.909 (0.366–2.067)	1.131 (0.58–2.134)	1.478 (0.627–3.336)
low Complement	1.45 (0.633–3.211)	1.106 (0.564–2.101)	1.342 (0.543–3.162)
anti-dsDNA	0.964 (0.446–2.073)	2.387 (1.232–4.824)	1.492 (0.648–3.555)
Anti-phospholipid antibodies	1.056 (0.316–3.125)	0.793 (0.293–1.945)	1.216 (0.308–4.2)
Proteinuria	0.628 (0.144–1.919)	1.056 (0.431–2.331)	0.37 (0.058–1.31)
Hematuria	0.999 (0.324–2.558)	0.987 (0.428–2.076)	0.749 (0.214–2.037)

ESR = erythrocyte sedimentation rate. Odds ratio for thrombocytopenia in anti-B83 positive patients could not be calculated since no anti-B83 positive patient had thrombocytopenia.



Supplementary figure 4 Part III | Graphical presentation of multivariate regression taking disease duration (diagnosis-blood sampling) and -activity as predictors and possibility of positive autoantibodies as outcome

Two activity stratified lines show the probability of having autoantibodies over the years of disease duration. Adjusted odds ratios for being autoantibody positive per one-year disease duration were **(a)** 0.98 (CI's= 0.95–1) **(b)** 0.97 (CI's= 0.95–0.99) and **(c)** 0.94 (CI's= 0.9–0.98). Adjusted odds ratio for being autoantibody positive in case of active SLE disease were **(a)** 1.43 (CI's= 0.9–2.27), **(b)** 1.67 (CI's = 1.03–2.68) and **(c)** 4.86, (CI's= 2.67–9.08).

Conclusion

To date, research has not fully explained the heterogenic pathogenic mechanisms contributing to the development of LN. Anti-C1q are considered to play a pathogenic role in LN because they exist in high concentrations in the glomeruli of LN patients and have a strongly negative predictive value for renal involvement.[91–94] However, exactly how anti-C1q contribute to disease activity and LN remain unclear, but this thesis provided considerable insights into the immunological effects of C1q/anti-C1q complexes on immune cells.

In the first project, I investigated the immunological effects of C1q/anti-C1q complexes in PBMCs in the presence of activated T cells. During this process, I adapted the *in vitro* model for anti-C1q-mediated autoimmunity previously published by our group[16,22,158], to study the direct effects of C1q/anti-C1q on T cells and PBMCs in general. Numerous earlier studies have proposed a direct effect of C1q on T cell activation and proliferation[13–15,54–59], but my findings demonstrated that both soluble and surface-bound C1q cannot replicate these effects due to differences in the experimental setting. Most studies have used soluble C1q, but I exposed T cells to surface-bound C1q. The two main reasons for this deviation were to allow anti-C1q binding and the subsequent formation of C1q/anti-C1q complexes and to avoid potential interference with T cell activating anti-CD3/CD28 antibodies. In the context of PBMCs, C1q/anti-C1q complexes elevated the release of pro-inflammatory cytokines. Specifically, activated T cells elicited a cell–cell contact-mediated increase in TNF and IFNγ secretion in monocytes. Furthermore, I discovered that the co-stimulatory pair CD40–CD154 is essential for the increased release of TNF in C1q/anti-C1q conditioned monocytes, which is dependent on the TRAF6 and JAK3-STAT5 signaling pathways (graphic summary in Figure 23). Thus, these results on cytokine levels – TNF, IL-10, and IFNγ – all supported the aberrant cytokine levels measured in sera from patients with active SLE[215,216] and provided evidence for how anti-C1q contribute to inflammation in LN (Rabatscher & Trendelenburg, 2022, published in Clinical & Translational Immunology).[217]

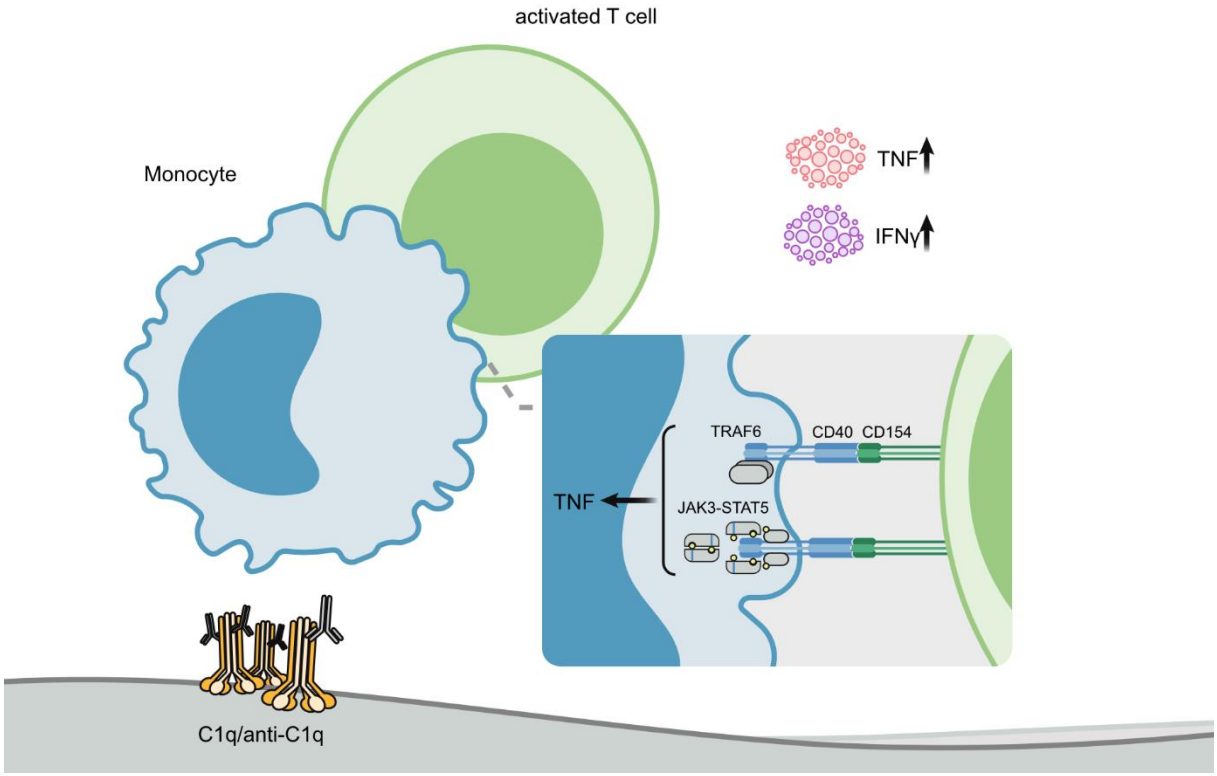


Figure 23 | Graphic summary of anti-C1q-mediated inflammation in PBMCs after T cell activation

Activated T cells elicit an increased TNF and IFNγ secretion in C1q/anti-C1q-conditioned monocytes. The release of TNF involved the co-stimulatory receptor–ligand pair CD40–CD154 and the intracellular signaling pathways TRAF6 and JAK3–STAT5.

In the second part of this thesis, I addressed the biosynthesis of C1q by HMDMs. Previous data from our group showed that C1q/anti-C1q complexes were capable of inducing *de novo* synthesis and releasing C1q in human macrophages *in vitro*.[22] To study the potential impact of C1q/anti-C1q complexes on the biosynthesis of C1q, I exploited our *in vitro* model of anti-C1q-mediated autoimmunity[16,22,158] and analyzed C1q protein levels after the inhibition of the protein synthesis at various stages (i.e., transcription, translation, and secretion) and mRNA C1q levels in HMDMs between different conditions by ELISA and qPCR, respectively. Confirming previous findings, C1q concentrations in the cell culture medium increased more in the presence of C1q/anti-C1q complexes than with only C1q and HSA.[22] However, inhibition of C1q synthesis in macrophages at any stage did not have a significant effect on the concentration of C1q in the cell culture medium. Additionally, the *C1QA* mRNA levels between differently conditioned macrophages were identical. To test the hypothesis of plate-derived C1q in the cell culture medium, biotin-labeled C1q was used to coat the plates and was later detected in the medium after cell culture, contrary to expectations. In summary, the results presented here do not support the

notion of freshly produced C1q in C1q/anti-C1q-conditioned HMDMs[22], instead promoting the release of plate-bound C1q into the vicinity.

The final part of this thesis covers the epitope-specificity of anti-C1q in SLE. A considerable amount of literature has highlighted the CLR as the primary target of anti-C1q.[23–26,85] However, the exact targets of the patient-derived polyclonal anti-C1q have not yet been identified. Therefore, the primary goal of this study was to identify the epitope-specificity of anti-C1q and to determine the association of epitope-specific anti-C1q with clinical parameters and disease manifestations by a high-resolution epitope-mapping approach and peptide-based ELISAs, respectively. In the epitope-mapping, we identified a total of six epitopes in the N-terminal CLR (three on the A-chain and three on the B-chain) present in SLE patient samples. A subsequent analysis of a large cohort by peptide-based ELISAs revealed that some epitope-specific autoantibodies associated with specific clinical features, whereas traditionally determined anti-C1q associated with a broader spectrum of clinical features and overall disease activity. Surprisingly, autoantibodies specific for the epitopes A09 and A15 were more accurate in discriminating between healthy donors and patients with SLE. Overall, anti-C1q comprise a valuable biomarker for SLE to monitor disease progression and activity as well as the potential to predict specific disease manifestations (Kleer *et al.*, 2022, published in Frontiers in Immunology).[144]

Thus, these presented findings yield novel information about the immunological consequences of C1q/anti-C1q complex formation in SLE. The complement system plays a key role in the pathophysiology of SLE and can thus be regarded as a bridge between innate and adaptive immunity. However, a better understanding of how C1q/anti-C1q complexes shape immune responses could produce new insights into multiple autoimmune diseases (e.g., SLE, HUVS, Sjögren's syndrome, rheumatoid vasculitis), potentially contributing to the development of new diagnostic and therapeutic approaches.

Outlook

Considering the growing body of knowledge on anti-C1q, C1q/anti-C1q can be considered to play a pivotal role in the development and progression of SLE. While with the work presented here, I could contribute to elucidating the immunological consequences of C1q/anti-C1q complexes, there remain open questions regarding the underlying mechanisms of anti-C1q in SLE.

Part I

Based on the assumption that anti-C1q interfere with C1q's ability to bind and interact with its intended binding partners, identification of the specific C1q receptor(s) in the involved cell populations could provide valuable information. Therefore, we intend to determine the potential receptor(s) for C1q in phagocytes, primarily HMDMs. A promising approach to identify potential receptors is the proteomic analysis of C1q/receptor pairs from phagocytes preincubated with C1q obtained from pull-down assays. Additionally, knowing the specific receptor(s) could potentially provide important insight into C1q-mediated effects on cellular functions, such as phagocytosis, differentiation, and cytokine responses. Although part of the anti-C1q mediated effects were observed to be Fcγ receptor-dependent[16], data comparing C1q/anti-C1q to alternative immune complexes (e.g., HSA/anti-HSA) revealed additional Fcγ receptor-independent effects. It is feasible that one of these Fcγ receptor-independent effects involves disturbance of C1q receptor interactions by anti-C1q. Such an effect could be investigated in *in vitro* experiments using receptor-specific monoclonal blocking antibodies (or Fab fragments), monoclonal anti-C1q (specific for a given epitope) or short C1q mimicking peptides to inhibit or provoke anti-C1q-mediated consequences. Furthermore, our group is currently in the process of exploring potential receptor candidates in HMDMs.

An alternative approach to enhance the knowledge of C1q/anti-C1q-mediated consequences could include single cell RNA sequencing of HMDMs in the presence of C1q and C1q/anti-C1q, respectively. Beyond this, the analysis of transcriptional data could produce valuable insights into alternatively modulated intracellular pathways associated with the observed cellular phenotype (e.g., attenuated phagocytosis, pro-inflammatory cytokine profile, and surface markers)[16] and subsequently connected to receptors initiating the intracellular pathways. This approach would yield additional information about differentiation and potential differences in cellular functions (e.g., cellular metabolism). However, the search for a C1q receptor by this approach would be more challenging than the initially mentioned proteomic analysis of C1q/receptor pairs, thus necessitating confirmation in additional *in vitro* experiments.

Additionally, all data presented in this part involve freshly obtained cells from healthy individuals. Future experiments could investigate the effects of T cell activation in the presence of C1q/anti-C1q in SLE-derived PBMCs. This approach would allow the translation of the presented results and ultimately provide insight into the situation in patient cells.

Part II

Regarding the detachment of C1q, it is unclear how the protein is released into the cell culture medium and to what extent anti-C1q contribute to this observation. A potential physiological and pathophysiological relevance of this detachment is intriguing. First, one could perform *in vitro* experiments with more physiological C1q targets than the polystyrene cell culture plate (e.g., apoptotic cells, immune complexes) to test for similar effects. Next, there are two possible explanations of how HMDMs could detach C1q from the plate surface into the cell culture medium. That is, this process could occur either indirectly through the internalization of the molecule or directly because of conformational changes induced by the binding of C1q receptors. To differentiate between these hypotheses, C1q labeled with a traditional or pH-sensitive (e.g., pHrodo™) fluorophore could be used to coat the cell culture plate followed by a subsequent analysis of the cells using flow cytometry (possibly ImageStream^X).

Furthermore, anti-C1q could facilitate the secretion or detachment of bound C1q, as highlighted by data from a previous study[22] and the current thesis, respectively. Although this study revealed that released C1q correlated with anti-C1q levels[22], no research has shown whether the quantity alone, epitope-specificity, or both determines the degree of detachment. Thus, one could assess a potential epitope-dependent effect by analyzing a number of monoclonal antibodies, ideally with known specificities, and test for concentration-depending effects of the monoclonal antibodies.

Lastly, anti-C1q are polyclonal IgG with a high affinity for neo-epitopes found on bound C1q. However, it is unclear whether C1q/anti-C1q dissociate or remain intact when detached from the target structure. To examine this condition, cell culture supernatants could be tested for C1q/IgG complexes using a sandwich ELISA that captures C1q and detects human IgG (similar to the previously described ELISA for circulating B cell activating factor/IgG complexes[218]).

Part III

The analysis of the epitope-specificity of anti-C1q facilitated the identification of peptide-specific antibodies associated with specific disease manifestations. These short linear peptides are not fully capable of reflecting C1q's complex structure composed of the 18 distinct polypeptide chains (i.e., C1qA, C1qB, and C1qC). However, these peptides allow one to test large cohorts with the potential for use in clinical application.

As mentioned in the publication, future confirmational investigations, with even larger sample sizes than used in our study, are required to confirm the results of this explorative study. Additionally, the repertoire of epitope-specific anti-C1q in patients with SLE could potentially change over the course of the disease. Thus, analyzing longitudinal data of the described peptide-specific antibodies could provide valuable information regarding disease progression.

Lastly, in our study we applied univariate logistic regression to all peptide candidates. Considering the large number of potential epitope-specific anti-C1q, a future approach could use a combination of multiple epitope-specific anti-C1q to associate with disease manifestations.

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Appendix

Manuscript I

Anti-C1q autoantibodies from systemic lupus erythematosus patients enhance CD40–CD154-mediated inflammation in peripheral blood mononuclear cells in vitro

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ORIGINAL ARTICLE

Anti-C1q autoantibodies from systemic lupus erythematosus patients enhance CD40–CD154-mediated inflammation in peripheral blood mononuclear cells *in vitro*

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Abstract

Objectives. Systemic lupus erythematosus (SLE) is a clinically heterogeneous autoimmune disease with complex pathogenic mechanisms. Complement C1q has been shown to play a major role in SLE, and autoantibodies against C1q (anti-C1q) are strongly associated with SLE disease activity and severe lupus nephritis suggesting a pathogenic role for anti-C1q. Whereas C1q alone has anti-inflammatory effects on human monocytes and macrophages, C1q/anti-C1q complexes favor a pro-inflammatory phenotype. This study aimed to elucidate the inflammatory effects of anti-C1q on peripheral blood mononuclear cells (PBMCs). **Methods.** Isolated monocytes, isolated T cells and bulk PBMCs of healthy donors with or without concomitant T cell activation were exposed to C1q or complexes of C1q and SLE patient-derived anti-C1q (C1q/anti-C1q). Functional consequences of C1q/anti-C1q on cells were assessed by determining cytokine secretion, monocyte surface marker expression, T cell activation and proliferation. **Results.** Exposure of isolated T cells to C1q or C1q/anti-C1q did not affect their activation and proliferation. However, unspecific T cell activation in PBMCs in the presence of C1q/anti-C1q resulted in increased TNF, IFN-γ and IL-10 secretion compared with C1q alone. Co-culture and inhibition experiments showed that the inflammatory effect of C1q/anti-C1q on PBMCs was due to a direct CD40–CD154 interaction between activated T cells and C1q/anti-C1q-primed monocytes. The CD40-mediated inflammatory reaction of monocytes involves TRAF6 and JAK3-STAT5 signalling. **Conclusion.** In conclusion, C1q/anti-C1q have a pro-inflammatory effect on monocytes that depends on T cell activation and CD40–CD154 signalling. This signalling pathway could serve as a therapeutic target for anti-C1q-mediated inflammation.

Keywords: anti-C1q autoantibodies, C1q, CD40, monocytes, systemic lupus erythematosus, T cells

INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with heterogeneous clinical manifestations and complex pathogenic mechanisms.[1] Antibodies against a wide range of autoantigens, formation of immune complexes and aberrant clearance of apoptotic cells are typical findings in patients with SLE.[2–4] The clearance of immune complexes and apoptotic cells involves the complement system, and deficits in molecules of the early classical pathway of complement (i.e. C1q, C1r, C1s, C4 and C2) are strongly associated with SLE.[5,6] Among those deficiencies, homozygous C1q deficiency is the strongest genetic risk factor for disease development.[7,8] However, primary C1q deficiency as a cause of SLE is rare. Most patients suffer from secondary hypocomplementemia, most likely caused by increased complement activation via the classical and lectin pathways associated with the occurrence of anti-C1q autoantibodies (anti-C1q).[9–12] These polyclonal, high-affinity autoantibodies recognise neo-epitopes expressed in the collagen-like region of bound C1q.[13–16] Furthermore, anti-C1q are associated with disease activity, particularly with severe lupus nephritis (LN). Patients with renal involvement show increasing levels of anti-C1q before a recurring exacerbation and high deposition of anti-C1q in glomeruli.[17–21] Considering a large number of functions of C1q[22] and the association of anti-C1q with SLE disease manifestations, it is highly likely that anti-C1q have a disease-modifying effect.[23,24] However, the exact means of how anti-C1q contribute to disease activity and LN remain unclear.

In addition to the role as an initiator protein of the classical pathway of complement and pattern recognition molecule, C1q also exerts cellular functions.[25] C1q bound to target patterns (e.g. apoptotic cells, pathogens and cholesterol crystals) facilitates phagocytosis and regulates a wide range of cytokines towards a less inflammatory cytokine secretion profile in human innate immune cells (e.g. monocytes, macrophages and immature dendritic cells).[26–31] Regarding anti-C1q, Thanei and Trendelenburg[32] demonstrated that C1q/anti-C1q complexes reverse the phagocytosis enhancing and anti-inflammatory effects of C1q and induce a pro-inflammatory phenotype in human monocyte-derived macrophages (HMDMs).

In addition, emerging evidence suggests that C1q exerts an immunosuppressive effect on innate immune cells and T cells.[33–38] Additionally, T cells have been implicated in SLE as, for example, they make up the majority of cells present in the tubulointerstitial lesions of kidney biopsies of SLE patients.[1,39] The direct interaction of soluble C1q and C1q receptors present on T cells results in less activation, fewer cell divisions and less cytokine secretion.[33,35,38] Furthermore, Clarke *et al.*[34] reported an indirect route for C1q to modulate T cell activation, proliferation and differentiation via macrophages primed with C1q-coated late apoptotic lymphocytes in *in vitro* co-culture experiments.

Taken together, T cells, macrophages, C1q and anti-C1q play an important role in the course of SLE. Nevertheless, the downstream mechanisms and functional consequences of C1q/anti-C1q complexes are still poorly understood. To better understand anti-C1q in SLE, we investigated the immunological effects of C1q/anti-C1q complexes on peripheral blood mononuclear cells (PBMCs) in a setting of activated T cells by studying cytokine secretion, T cell proliferation and activation and monocyte surface marker expression.

RESULTS

Anti-C1q bound to C1q increases cytokine secretion in PBMCs after T cell activation

We first analysed the effect of C1q/anti-C1q complexes on PMBCs in a non-septic chronic inflammatory setting. For this purpose, PBMCs were cultured for 24 h on bound C1q preincubated with anti-C1q negative sera (C1q/NHS) from healthy donors and bound C1q preincubated with anti-C1q positive sera from SLE patients (C1q/anti-C1q).

To induce an inflammatory milieu, PBMCs were simultaneously activated by a dose of 5 μL mL^{-1} soluble human CD3/CD28 T cell activator. In contrast to the commonly used surface or bead-bound anti-CD3/CD28 T cell activators, there was no interference between C1q and the soluble tetrameric complex structure used in our *in vitro* model (Supplementary figure 1a and b). Additionally, the presence of human serum in PBS 1 M NaCl (deficient in calcium and magnesium) did not lead to the activation of the complement cascade and potential attachment of complement

onto the plate as shown by the lack of C3 deposition (Supplementary figure 1c).

PBMCs significantly upregulated TNF ($P < 0.0001$), IFN-γ ($P < 0.0001$) and IL-10 ($P = 0.0003$) secretion in the presence of C1q/anti-C1q complexes obtained from 20 SLE patients compared with C1q/NHS (Figure 1, left). Additionally, levels of anti-C1q in SLE patients were found to correlate with TNF ($r = 0.6592$), IFN-γ ($r = 0.6349$) and IL-10 ($r = 0.5226$) concentrations (Figure 1, right). Intra-patient comparison of anti-C1q negative (< 50 AU) and anti-C1q positive (≥ 50 AU) sera from separate time points revealed equivalent increases in TNF, IFN-γ and IL-10 as shown in C1q/NHS and C1q/anti-C1q (Supplementary figure 2). Based on this observation, further experiments elucidating involved cell types and cellular mechanisms were standardised to the use of anti-C1q-positive plasma from a previously published patient with 1000 AU anti-C1q.[40]

Because the effect of the C1q/anti-C1q complexes might be solely attributed to the interaction of IgG and Fc receptors on monocytes, we exposed PBMCs to coated monomeric IgG (5 μg mL^{-1}) of anti-C1q-negative and anti-C1q-positive samples, respectively. Interestingly, the presence of purified anti-C1q-positive IgG alone did not increase TNF secretion compared with anti-C1q-negative IgG or C1q alone, whereas purified anti-C1q-positive IgG complexed with C1q elevated TNF secretion (Supplementary figure 3a and b). As a control for the specificity of the C1q/anti-C1q complexes, an alternative immune complex consisting of HSA/anti-HSA was also tested. Again, TNF secretion in PBMCs did not significantly differ between HSA/anti-HSA complexes and bound HSA alone (Supplementary figure 3c).

Bound C1q does not affect T cells directly

Previous studies suggest a direct anti-proliferative and anti-inflammatory effect of soluble C1q on T cells.[33,35,38,41] To investigate the potential direct effect of bound C1q and C1q/anti-C1q complexes, respectively, on T cell activation and proliferation, we incubated isolated CD3$^+$ T cells with bound HSA (1 μg per well), bound C1q (1 μg per well), C1q/anti-C1q complexes and uncoated wells in the presence of soluble C1q (100 μg mL^{-1}). Bound C1q did not significantly modulate the secretion of TNF and IL-10 as well as the expression of activation markers CD25 and CD69 after 24 h of T cell activation when compared to HSA (Figure 2a–d). However, TNF secretion was significantly decreased in the presence of soluble C1q compared with HSA after 24 h (Figure 2a). No significant difference in T cell proliferation was observed between bound HSA, bound C1q, bound C1q/anti-C1q and soluble C1q exposure after 96 h (Figure 2e and f, gating strategy in Supplementary figure 4b).

The presence of CD14$^+$ cells is essential for increased TNF secretion in the presence of C1q/anti-C1q complexes

We next performed an intracellular cytokine staining after 24 h of anti-CD3/CD28 stimulation to evaluate the source of the observed cytokines. Categorisation of CD4, CD8, CD14, CD19, CD56 and other cell types revealed that the main TNF and IL-10 producing cells are CD14$^+$ monocytes (TNF: $P < 0.001$; IL-10: $P = 0.017$) when exposed to C1q/anti-C1q compared with C1q/NHS (Supplementary figure 5b). With these results and the fact that a previous study on HMDMs found a pro-inflammatory cytokine secretion profile in the presence of C1q/anti-C1q complexes,[32] we next investigated whether the interaction between monocytes and activated T cells accounts for the observed increase in TNF, IFN-γ and IL-10. For this, we performed autologous co-culture experiments of isolated CD3$^+$ T cells and CD14$^+$CD16$^-$ monocytes with concomitant anti-CD3/CD28 stimulation of T cells for 24 h. Unlike IL-10 secretion, the increase in TNF and IFN-γ in the co-culture setting after exposure to C1q/anti-C1q complexes was identical to the observation made in PBMCs, suggesting that monocytes are essential for the secretion of these cytokines (Figure 3a and b, left and middle panel). In contrast, IL-10 secretion seems to require the interaction of further immune cells (Figure 3c, left and middle panel). In line with this finding, depletion of CD14$^+$ cells (89–95% efficiency) abolished the pro-inflammatory effect for IFN-γ ($P = 0.625$) and greatly reduced the increase observed in TNF ($P = 0.031$) and IL-10 secretion ($P = 0.031$) (Figure 3, right panel), suggesting that the

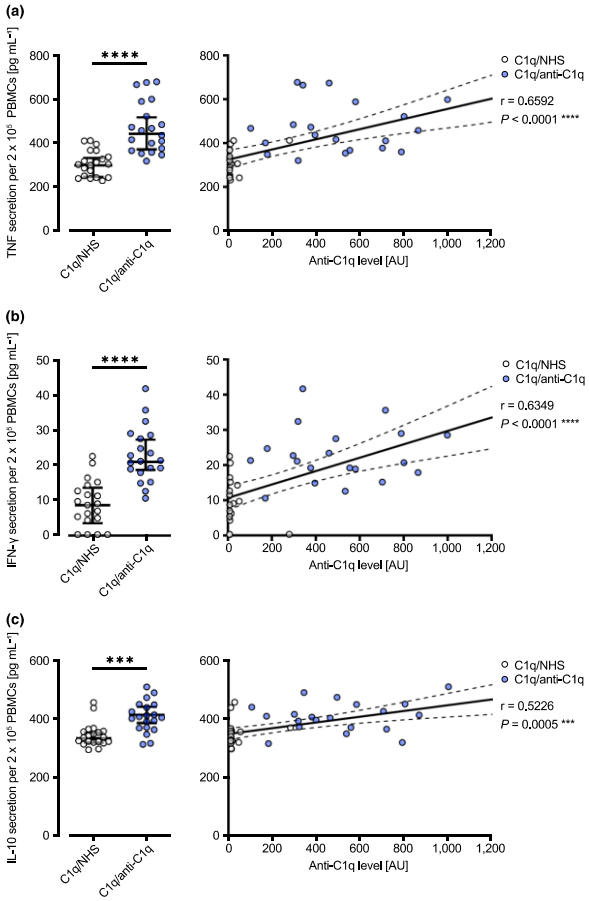

Figure 1. Increase in TNF, IFN-γ and IL-10 secretion in peripheral blood mononuclear cells (PBMCs) after T cell activation correlates with anti-C1q levels. PBMCs activated by tetrameric anti-CD3/CD28 complexes were cultured on C1q preincubated with the serum of 20 systemic lupus erythematosus patients (C1q/anti-C1q) or with the serum of 20 healthy donors (C1q/NHS) for 24 h. Cell culture supernatants were analysed for **(a)** TNF, **(b)** IFN-γ and **(c)** IL-10 secretion by ELISA. Data points represent the median cytokine concentration obtained from PBMCs of four unrelated healthy donors from independent experiments exposed to one single serum sample. (Left) Horizontal lines with error bars show median with IQR. Mann–Whitney *U*-test, ***$P < 0.001$, ****$P < 0.0001$. (Right) Solid line represents linear regression with dotted lines indicating the 95% confidence bands. Spearman's rank correlation of cytokine secretion and anti-C1q levels, ***$P < 0.001$, ****$P < 0.0001$.

remaining $CD14^+$ monocytes are sufficient for a moderate but still significant increase in TNF and IL-10.

Taken together, the interaction between activated T cells and monocytes is responsible for increased TNF and IFN-γ levels in the presence of C1q/anti-C1q complexes, whereas increased IL-10 levels require further signals from cells present in PBMCs but missing in the co-culture of monocytes and T cells.

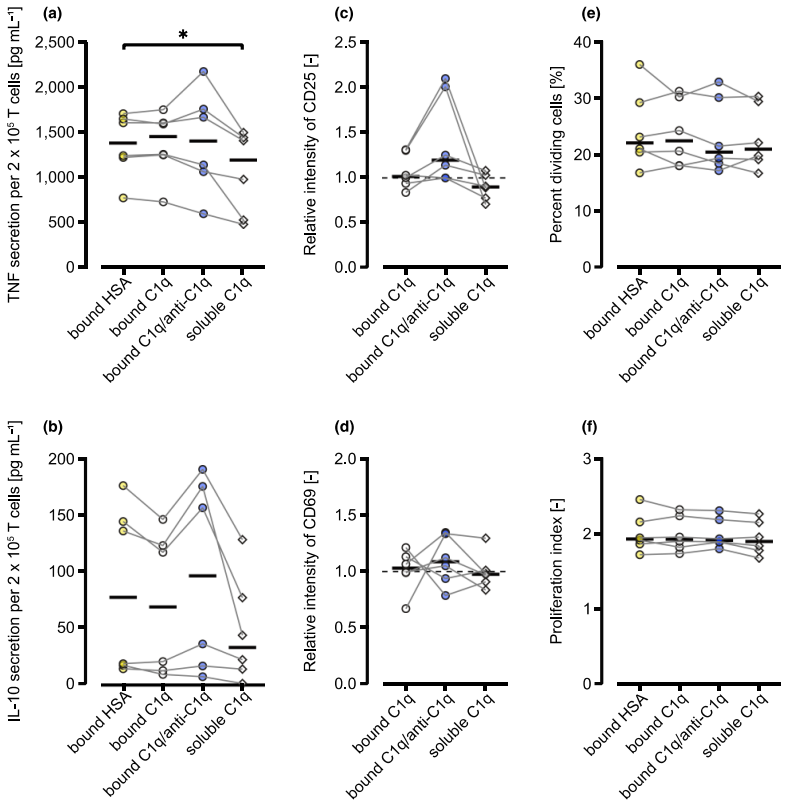

Figure 2. T cell proliferation, activation and IL-10 secretion in activated T cells are not affected by bound C1q, bound C1q/anti-C1q and soluble C1q, respectively, whereas TNF secretion is decreased in the presence of soluble C1q. T cells were isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors and cultured on bound HSA, bound C1q, bound C1q preincubated with anti-C1q-positive systemic lupus erythematosus serum (bound C1q/anti-C1q), or together with soluble C1q without coating. T cells were activated by tetrameric anti-CD3/CD28. Cytokines **(a)** TNF and **(b)** IL-10 as well as activation markers **(c)** CD25 and **(d)** CD69 were analysed after 24 h by ELISA and flow cytometry, respectively. For proliferation assessment, cells were stained with CFSE prior to the experiment and **(e)** per cent dividing cells and **(f)** proliferation index were analysed by flow cytometry after 96 h. Data points represent six different healthy donors used to obtain PBMCs analysed in independent experiments with connecting lines linking data points of a single individual. Median values are shown as solid horizontal lines. The Friedman test with Dunn's posttest correction (all vs bound HSA), $*P < 0.05$. **(c, d)** Relative intensity is calculated by normalising MFI of bound C1q, bound C1q/anti-C1q and soluble C1q to bound HSA. The horizontal dashed line marks the relative change in intensity of 1.0 (Supplementary figure 4a depicts the gating strategy).

Direct cell–cell contact between monocytes and T cells mediates TNF secretion through CD40–CD154 binding

To evaluate whether the increased TNF concentration in co-cultured monocytes and T cells requires direct cell–cell contact or is mediated via soluble factors, we expanded the co-culture to a transwell experiment. For this, monocytes were exposed to C1q/NHS and C1q/anti-C1q coatings, whereas culturing and activation with anti-CD3/CD28 of T cells for 24 h occurred in inserts

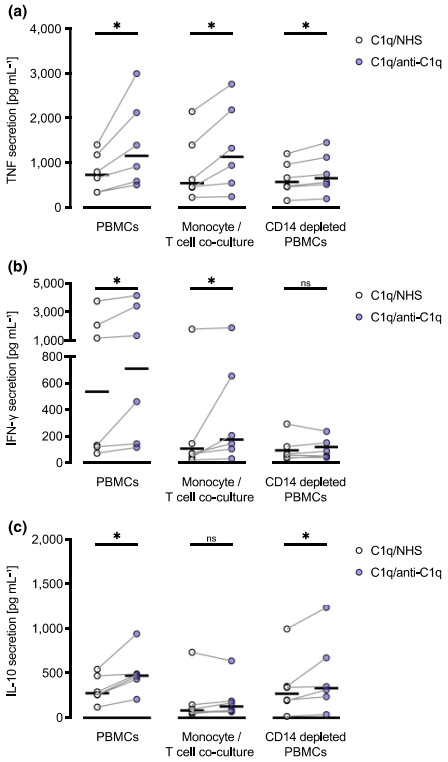

Figure 3. Presence of $CD14^+$ cells is essential for the increased cytokine secretion in the presence of C1q/anti-C1q complexes. Peripheral blood mononuclear cells (PBMCs), monocytes and T cells (co-culture 1:5 ratio) and CD14-depleted PBMCs (89–95% efficacy) were cultured on C1q preincubated with anti-C1q-negative NHS (C1q/NHS) or anti-C1q-positive systemic lupus erythematosus serum (C1q/anti-C1q) and activated by tetrameric anti-CD3/CD28 complexes for 24 h. Cell culture supernatants were analysed by ELISA for **(a)** TNF, **(b)** IFN-γ and **(c)** IL-10 secretion. Median cytokine concentrations are shown as horizontal lines, and data points represent independent experiments analysing six different healthy donors used to obtain PBMCs with connecting lines linking data points of a single individual. The Wilcoxon matched-rank test, $*P < 0.05$; ns, not significant.

separated from the monocytes and coatings. Differences in TNF secretion between C1q/NHS and C1q/anti-C1q settings disappeared after the separation of monocytes and T cells, indicating that cell–cell contact is required (Figure 4a).

Next, we aimed to assess surface markers on monocytes present in the immune synapse of monocytes and T cells. Therefore, we analysed CD40, CD80 and CD86 expression on $CD11c^+$ cells after T cell activation in PBMCs. After 24 h, CD80 and CD86 levels did not differ in $CD11c^+$ cells between C1q/NHS and C1q/anti-C1q coatings (CD80: $P = 0.562$, CD86: $P = 0.688$). However, CD40 was slightly downregulated in the presence of C1q/anti-C1q complexes compared with C1q/NHS (CD40: $P = 0.031$) (Figure 4b, gating strategy in Supplementary figure 4a).

Previous studies showed the importance of the CD40–CD154 interaction in T cell-mediated immune responses and activation of macrophages.[42–45] Therefore, we explored this interaction in our setting. For this purpose, PBMCs

were cultured and T cells activated as described above in the presence of either a mouse anti-CD154 blocking antibody (5 μg mL^{-1}; clone 24–31) or an isotype control (5 μg mL^{-1}; clone P3.6.2.8.1). The addition of the CD154 blocking antibody resulted in a decrease in TNF secretion compared with the isotype control antibody and the disappearance of a significant difference in TNF secretion between C1q/NHS and C1q/anti-C1q priming ($P = 0.094$) (Figure 5a, right panel).

Considering that the inhibition of CD154 normalised TNF secretion caused by the presence of C1q/anti-C1q, we next assessed whether the CD40 signalling in monocytes is sufficient for the observed differences in TNF secretion. For this purpose, isolated monocytes were cultured on C1q/NHS or C1q/anti-C1q. Since unstimulated monocytes express only very low levels of CD40 (data not shown) compared with PBMCs with activated T cells (Figure 4b), additional priming with 500 U mL^{-1} IFN-γ for 18 h was necessary to achieve comparable CD40 levels in isolated monocytes.[46] Afterwards, CD154-expressing rhabdomyosarcoma (RD) cells were added in a 2:1 ratio (monocytes/RD cells) for 24 h to activate monocytes and mimic activated CD154-expressing T cells. CD154-stimulated monocytes cultured on C1q/anti-C1q increased TNF secretion compared with monocytes on C1q/NHS ($P = 0.008$), confirming the CD40–CD154 interaction to be an important signal for TNF secretion after exposure of monocytes to C1q/anti-C1q complexes (Figure 5b). In a controlled setting with non-transfected RD cells, monocytes

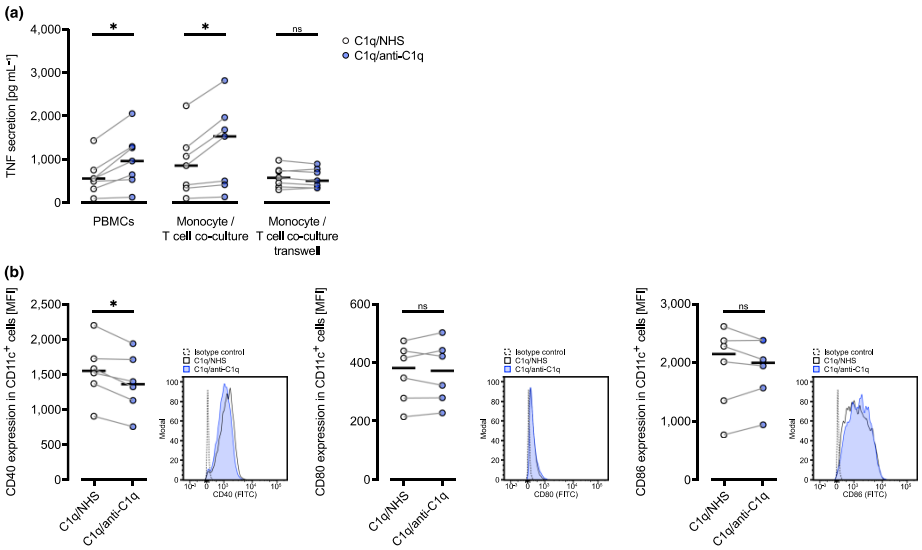

Figure 4. Increase in TNF secretion after exposure to C1q/anti-C1q complexes requires cell–cell contact between monocytes and T cells and involves CD40 downregulation in CD11^{+} cells. **(a)** Peripheral blood mononuclear cells (PBMCs) or monocytes and T cells (1:5 ratio) co-cultured either together or separated by 0.4 μm pore polyester membrane inserts (monocytes in the receiver plate, T cells in the permeable support system) were exposed to bound C1q, which was preincubated with anti-C1q-negative NHS (C1q/NHS) or anti-C1q-positive systemic lupus erythematosus serum (C1q/anti-C1q). Cells were activated with tetrameric anti-CD3/CD28 complexes for 24 h. Cell culture supernatants were analysed for TNF secretion by ELISA. Median cytokine concentrations are shown as horizontal lines, and data points represent seven different healthy donors analysed in independent experiments. **(b)** Analyses of CD40, CD80 and CD86 in CD11c^{+} cells were performed by flow cytometry after 24 h of cell culture. Median MFIs are shown as horizontal lines, and data points represent six different healthy donors analysed in independent experiments. Connecting lines link data points of a single donor used to obtain cells. The Wilcoxon matched-rank test, $*P < 0.05$; ns, not significant. Flow cytometry histograms show one donor representative for six healthy donors (Supplementary figure 4b depicts the gating strategy).

did not secrete detectable levels of TNF (data not shown).

In summary, our data demonstrate that despite the slight downregulation of CD40, the CD40–CD154 signalling axis is sufficient for the upregulation of TNF secretion in monocytes that encountered C1q/anti-C1q complexes.

JAK3-STAT5 and TRAF6 are partially redundant intracellular CD40 signalling pathways responsible for TNF secretion in anti-C1q-primed monocytes

Intracellular CD40 signalling is divided into tumour necrosis factor receptor-associated factors (TRAF) dependent and independent signalling, including the JAK3-STAT5 pathway.[47] Both can participate in the induction of TNF in monocytes.[48]

To assess intracellular pathways in our *in vitro* autoimmune model, we co-cultured IFN-γ-primed isolated monocytes and CD154-expressing RD cells as described before. However, before the addition of CD154-expressing RD cells, monocytes were treated with either the JAK3 inhibitor PF-06651600 (0–10 μM), TRAF6 inhibitor 6877002 (0–20 μM) or NF-κB inhibitor JSH-23 (0–30 μM) for 4 h. Additionally, combinations of PF-06651600 (0–10 μM) plus TRAF 6 inhibitor 6877002 (20 μM) or JSH-23 (30 μM) or a combination of PF-06651600 (0–10 μM) plus TRAF 6 inhibitor 6877002 (20 μM) and JSH-23 (30 μM) were applied to detect potential cumulative effects.

All three inhibitors blocked TNF secretion dose-dependently, achieving an approximately 42–54% reduction at most (Figure 6a–c). Interestingly, the combination of the JAK3 inhibitor PF-06651600 and the TRAF 6 inhibitor 6877002 further decreased TNF secretion to 27% of the baseline (Figure 6d), whereas the addition of the NF-κB inhibitor JSH-23 to PF-06651600 did not further decrease TNF secretion (Figure 6e and f).

These data demonstrate that TNF secretion in CD40-activated IFN-γ-primed monocytes is mediated by multiple and partially additive intracellular signalling pathways, with TRAF6 and JAK3-STAT5 signalling being the least redundant.

DISCUSSION

Anti-C1q are considered to play a pathogenic role in the development and maintenance of SLE.[23,24] Anti-C1q correlate with disease activity and can be found in C1q/anti-C1q complexes in the glomeruli of SLE patients with severe LN.[17,21,49] However, the pathogenic impact of anti-C1q and C1q/anti-C1q complexes, in particular, on the disease is not well defined. Accumulating evidence indicates

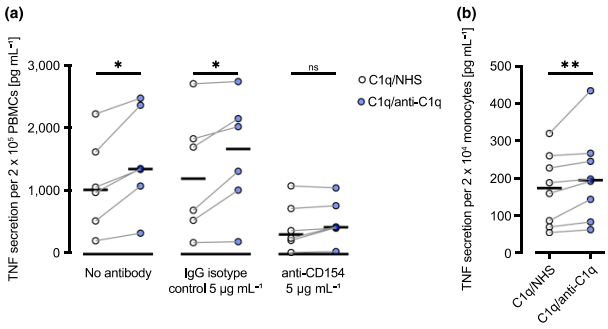

Figure 5. CD40 signalling to monocytes is essential for increased TNF secretion after exposure to C1q/anti-C1q complexes. **(a)** Peripheral blood mononuclear cells (PBMCs) from healthy donors were cultured in the presence of C1q, which was preincubated with anti-C1q-negative NHS (C1q/NHS) or anti-C1q-positive systemic lupus erythematosus (SLE) serum (C1q/SLE) for 24 h. The addition of a blocking mouse anti-CD154 IgG antibody (5.0 μg mL^{-1}) showed a decrease in TNF secretion compared with the isotype control (5 μg mL^{-1}). **(b)** CD40–CD154 signalling to monocytes was confirmed by co-culturing IFN-γ-primed (500 U mL^{-1}, 18 h) monocytes to CD154-expressing RD cells in the presence (C1q/anti-C1q) or absence (C1q/NHS) of C1q/anti-C1q complexes for 24 h. Cell culture supernatants were analysed for TNF secretion by ELISA. Median cytokine concentrations of TNF are shown as horizontal lines. Data points represent **(a)** six and **(b)** eight different healthy donors analysed in independent experiments. Connecting lines link data points of a single donor used to obtain PBMCs. The Wilcoxon matched-signed test, $*P < 0.05$, $**P < 0.01$; ns, not significant.

that kidney damage is not solely caused by (auto-) antibodies but involves immune cells, including myeloid, T, NK and B cells[50] present in the glomeruli of LN biopsies as mediators of direct tissue damage.[51–53] In addition, neutrophils in SLE patients were found to have an active transcription signature and to be capable of interacting with deposited immune complexes (i.e. Fc region of IgG), which include C1q/anti-C1q, as well as contributing to complement activation.[54–56]

Our study focused on the cellular response in PMBCs downstream of anti-C1q. We found that C1q/anti-C1q complexes induce a pro-inflammatory cytokine response – TNF, IFN-γ and IL-10 – in PBMCs in a setting of unspecific aseptic inflammation. Moreover, CD154-mediated CD40 signalling in monocytes was discovered to be involved in the C1q/anti-C1q related increase in TNF.

Generally, autoantibodies and immune complexes are believed to be the primary drivers of SLE. However, aberrant cytokine levels, such as IL-6, IL-10, IL-17, TNF and IFN-γ, are commonly observed in SLE patients.[57–62] Besides their effects on differentiation, maturation and activation of immune cells, cytokines are involved in local inflammatory responses and tissue injury. Additionally, an array of cytokines can be used to monitor disease activity and predict disease severity.[58] In the context of LN, abundant levels of TNF, IL-10 and IFN-γ, as well as a simultaneous accumulation of anti-C1q in kidneys of SLE patients with renal involvement, have been observed, suggesting local synthesis of these particular cytokines.[21,49,63–65] In line with previous studies on C1q/anti-C1q complexes and their immunological effects on HMDMs,[32] we found a C1q/anti-C1q-mediated increase in TNF and IFN-γ in PBMCs of healthy donors after unspecific mild T cell activation. In contrast to HMDMs, IL-10 was also elevated in our experimental setting and reflected the situation in the serum of SLE patients with active disease.[66,67]

Concerning peripheral tolerance, T cell dysregulation is described as important in forming autoantibodies and the pathogenesis of SLE in general.[68] Expression of surface C1q receptors (i.e. gC1qR and cC1qR) in T cells suggests the capability to interact with C1q, which may affect T cell functions directly.[33,35,38] In fact, data from previous studies show immunoregulatory effects, such as reduced proliferation, activation and effector functions, upon C1q binding.[33,35,38] Interestingly, the binding of the collagen-like and globular heads region to their respective receptors is described as responsible for C1q's effects on T cells.[33,35,38] Contrary to earlier findings, our data on the direct impact of C1q on T cells do not demonstrate the same immunoregulatory effects. However, the experimental settings in the mentioned studies differ fundamentally. Our *in vitro* model of anti-C1q-mediated autoimmunity uses small amounts of surface-bound C1q, whereas models used in previous studies investigated large amounts of soluble C1q in the cell culture medium. This important difference was introduced in our study to overcome two major challenges. First, allowing anti-C1q to bind C1q and thus enable the formation of immune complexes that require the exposure of cryptic epitopes being exposed on bound C1q.[69] Second, avoiding the potential interaction of soluble C1q with aggregated stimulating anti-CD3/CD28 antibodies could neutralise the activator and thus lead to misleading results. Interestingly, a study on T cells from C1q-deficient autoimmune-prone mice supports our observations regarding the proliferation and activation of human T cells.[70]

The binding of CD154 and its receptor CD40 are crucial for adaptive immunity and the pathogenic processes observed in SLE, including B cell proliferation and differentiation.[47] Furthermore, both surface molecules represent promising therapeutic targets, as shown by the recent developments of the inhibiting anti-CD154 (Fab') fragment dapirolizumab pegol and the anti-CD40 antibody iscalimab, both being in clinical phase II and phase III trials, respectively.[71,72] Mostly described as a costimulatory factor in B cells, CD40 is a potent pro-inflammatory signalling pathway in monocytes and macrophages capable of inducing the synthesis of TNF and IL-1β.[43,73] These findings concur well with the induction of TNF in our co-culture experiments with monocytes and CD154-expressing RD cells and the decreased secretion of TNF upon CD154 inhibition. We could determine that cell–cell contact between C1q/anti-C1q-primed monocytes and activated T cells is crucial and largely dependent on CD40–CD154 ligation for inflammatory cytokine secretion.

Contrary to expectations, CD40 surface expression in $CD11c^{+}$ cells was slightly decreased in the presence of C1q/anti-C1q compared with exposure to C1q alone. Previous studies show

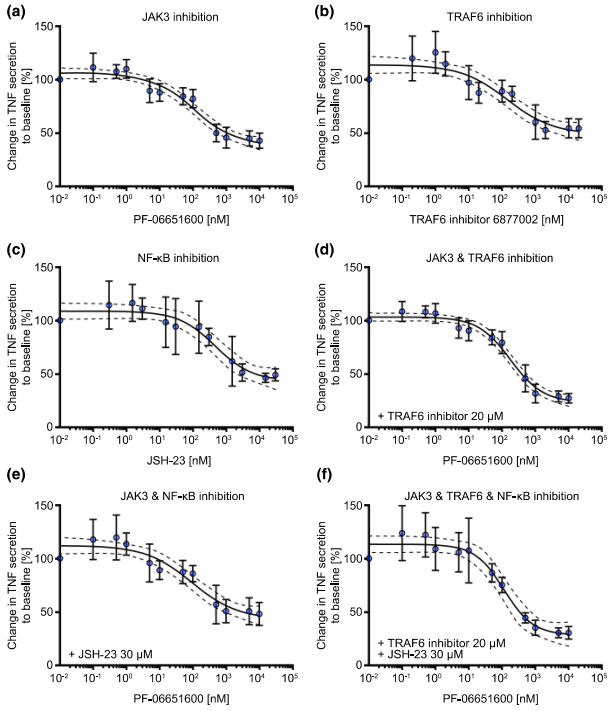

Figure 6. TNF secretion occurs via partially redundant intracellular CD40 signalling pathways JAK3-STAT5 and TRAF6. Monocytes were isolated from Peripheral blood mononuclear cells (PBMCs) of healthy donors and cultured in the presence of C1q/anti-C1q. Cells were preincubated with 500 U mL^{-1} IFN-γ for 18 h. Prior to the addition of CD154-expressing RD cells, monocytes were treated with **(a)** PF-06651600 0–10 µM (JAK3 inhibitor), **(b)** TRAF6 inhibitor 6877002 0–20 µM, **(c)** JSH-23 0–30 µM (NF-κB inhibitor), **(d)** PF-06651600 0–10 µM plus TRAF6 inhibitor 6877002 20 µM, **(e)** PF-06651600 0–10 µM plus JSH 30 µM and **(f)** PF-06651600 0–10 µM plus TRAF6 inhibitor 6877002 20 µM and JSH-23 30 µM for 4 h. Cell culture supernatants were analysed for TNF secretion by ELISA after 24 h of monocyte/RD cell co-culture. Data points represent mean inhibition of TNF secretion, normalised to the secretion without the addition of any inhibitor, of **(a–e)** six and **(f)** four different healthy donors. Error bars show standard deviations, solid lines show a four-parametric nonlinear regression, and dashed lines show the 95% confidence bands.

reduced CD40 expression and synthesis of pro-inflammatory cytokines in monocytes in the presence of IL-10 and IL-4.[74,75] Therefore, we hypothesise that this downregulation of CD40 is the result of a negative feedback mechanism caused by the increased IL-10 levels observed in our model. Next, we sought to investigate the responsible intracellular pathways in monocytes leading to TNF induction. CD40 signalling in monocytes is complex and comprises several pathways, including TRAF-dependent and independent (i.e. JAK3-STAT5) pathways.[47] In line with previous studies, we describe two partially redundant signalling pathways, TRAF6 and JAK3-STAT5, responsible for TNF secretion downstream of C1q/anti-C1q.[48,76]

We are well aware that our study has some limitations. The first is the simplified *in vitro* model used in our study, which is likely to only partially reflect the complex events *in vivo*, including the possible role of other immune cells (i.e. neutrophils and B cells). Notably, the surface characteristics of a tissue culture-treated plate to which C1q was attached to enable anti-C1q binding are probably different from biological surfaces. Furthermore, C1q's conformation closely depends on the target

structure, affecting the exposure of neo-epitopes that allow anti-C1q binding.[77] However, plate-bound C1q allows anti-C1q binding that correlates with disease activity, as determined in many clinical studies.[17,78,79] In addition, our study on anti-C1q induced cytokine secretion is well in line with findings of the previous human *in vitro* studies, as well as the cytokine profile found in serum and kidney samples of patients with active SLE.[32,58] Additionally, instead of monoclonal anti-C1q, we used polyclonal high-affinity patient-derived anti-C1q antibodies for our analyses. Lastly, our *in vitro* model replaced the toll-like receptor 4 stimulant lipopolysaccharide with CD3/CD28 targeting antibody complexes. Not only does this adaptation result in an aseptic inflammatory setting, but it also shows that active T cells can trigger anti-C1q-mediated inflammatory pathways.

In conclusion, in this study, we describe the immunological effects of anti-C1q on PBMCs that depend on unspecific T cell activation. Our findings reveal that C1q/anti-C1q complexes upregulate TNF, IFN-γ and IL-10. TNF and IFN-γ secretion from monocytes requires direct interaction with T cells, whereas IL-10 secretion from monocytes depends on further signals not provided in the co-culture of monocyte and T cell. Most notably, CD40 signalling in C1q/anti-C1q-primed monocytes is essential for TNF production and could serve as a therapeutic target for anti-C1q-mediated inflammation.

METHODS

Cell culture

Peripheral blood mononuclear cells

Peripheral blood from healthy donors was collected in ethylenediaminetetraacetic acid tubes at the Blood Transfusion Center of the University Hospital Basel (Basel, Switzerland). PBMCs were isolated by density gradient centrifugation using Lymphoprep (Serumwerk, Bernburg, Germany).

Monocytes

$CD14^+CD16^-$ monocytes were obtained from PBMCs by immunomagnetic negative selection (EasySep™ Human Monocyte Isolation Kit; Stemcell Technologies, Vancouver, BC, Canada), according to the manufacturer's instruction (yielding an average purity of 85–92% viable $CD14^+$ cells in our experiments as determined by flow cytometry). To induce CD40 expression for monocyte/RD cell co-culture experiments, isolated monocytes were preincubated with 500 U mL^{-1} IFN-γ (PeproTech, Cranbury, NJ, USA) in a complete cell culture medium [RPMI supplemented with 300 mg mL^{-1} L-glutamine, 25 mM HEPES, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10% (v/v) foetal calf serum (all from Life Technologies, Carlsbad, CA, USA)] for 18 h.

T cells

Similarly, an immunomagnetic negative selection was used to isolate T cells from PBMCs (EasySep™ Human T Cell Isolation Kit; Stemcell Technologies), according to the manufacturer's instruction (yielding an average purity of 90–95% $CD3^+$ viable cells as determined by flow cytometry).

CD14-depleted PBMCs

To deplete $CD14^+$ cells from PBMCs, an immunomagnetic positive selection kit for $CD14^+$ (CD14 MicroBeads, human; Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturer's instruction leading to an average depletion rate of 89–95% in our experiments (determined by flow cytometry).

RD cells

Human-derived TE671 RD cells from ATCC (American Type Culture Collection; LGC, Wesel, Germany) non-transfected and stably transfected with human CD154 (a kind gift from Nicholas Sanderson, Laboratory of Clinical Neuroimmunology, Department of Biomedicine, University of Basel, Basel, Switzerland)[80] were cultured in complete cell culture medium in cell culture bottles (BD Biosciences, Franklin Lakes, NJ, USA) at 37°C and 5% CO_2. Medium renewal and subculturing were performed in a ratio of 1:10 every 3–4 days.

Anti-C1q/IgG source

The selection of 20 sera/plasma from SLE patients included in the Swiss Systemic Lupus Erythematosus (SSCS) was based on biomaterial availability, fulfilling at least three of the 11 criteria of the American College of Rheumatology,[81,82] and anti-C1q levels (100–1000 AU). Of the 20 patients, 16 (80%) were female, and four (20%) were male. The median age at the time of blood sampling was 42 (27.5–40.3) years. Normal human serum (NHS) was obtained from age and sex-matched healthy blood donors from the Blood Transfusion Center of the University Hospital Basel.

To determine cellular mechanisms, anti-C1q-positive plasma was obtained from a previously described 20-year-old female SLE patient with active class IV LN at the time of sampling, fulfilling six of the 11 American College of Rheumatology criteria.[40] Anti-C1q levels in this individual were quantified by a previously described anti-C1q ELISA (1000 AU, cut-off value 50 AU)[15,16,83] and confirmed by a commercially available anti-C1q ELISA kit (Bühlmann, Schönenbuch, Switzerland) used in our clinical routine laboratory (2599 U mL^{-1}, cut-off 15 U mL^{-1}). For comparison, anti-C1q-negative (< 5 AU) plasma of an age-matched healthy female donor was

included as a negative control. The local Ethics Committee approved the sampling and use of blood samples included in this study (EKZ No. 110/04; 130/05).

In vitro model of anti-C1q autoimmunity

The *in vitro* model of anti-C1q autoimmunity was used as described before.[32,84,85] Briefly, flat-bottom 96-well plates (Eppendorf, Hamburg, Germany) were coated with 70 μL of 5 μg mL^{-1} purified human C1q (Complement Technology, Tyler, TX, USA) in coating buffer (0.4 M sodium carbonate buffer, pH 9.6) overnight at 4°C. The plates were washed twice with 140 μL PBS (Life Technologies) before adding anti-C1q-positive (SLE) or anti-C1q-negative (NHS) sera. Each serum sample was centrifuged at 14 000 *g* at 4°C for 30 min and diluted at 1:100 in PBS 1 M NaCl (Sigma-Aldrich, St. Louis, MO, USA) before incubation on a shaker (500 rpm) at room temperature for 1 h. Again, plates were washed four times with 140 μL PBS. Next, PBMCs (200 000 per well), T cells (200 000 per well) or monocytes/T cells (20 000 monocytes and 100 000 T cells per well) were added and activated with 5 μL mL^{-1} soluble tetrameric anti-CD3/anti-CD28 complex (ImmunoCult™ Human CD3/CD28 T cell activator; Stemcell Technologies) in a volume of 200 μL for 24 h.

T cell proliferation

To assess T cell proliferation, T cells were labelled with 5 μM CFSE (Biolegend, San Diego, CA, USA) at 37°C for 10 min and quenched five times with a complete cell culture medium. T cell proliferation was analysed by flow cytometry after 96 h. Per cent dividing cells and the proliferation index were calculated by FlowJo 10.7.1 (BD Biosciences) and used to describe T cell proliferation.[86]

Cytokine quantification

Cell culture supernatants were collected after the indicated experiment period, centrifuged (1000 *g*, 4°C, 10 min) to remove cell debris and stored at −80°C until further quantification. Commercially available ELISA kits for TNF (BD Biosciences), IL-10 (Biolegend) and IFN-γ (Immunotools, Friesoythe, Germany) were used to measure cytokine concentrations according to the manufacturer's instructions.

Anti-C1q quantification

Anti-C1q ELISA was performed as previously published.[9,16,83,87] In brief, ELISA plates were coated with 5 μg mL^{-1} purified human C1q. Blood samples were diluted at 1:50 in high-salt buffer [PBS 1 M NaCl with 0.05% Tween 20 (Sigma-Aldrich)] and added to the C1q-coated wells for 1 h at 37°C. To detect bound IgG, alkaline phosphatase-conjugated rabbit anti-human IgG (Promega, Madison, WI, USA) was used. Absorbance at 405 nm was read using a microplate ELISA reader (BioTek Instruments, Winooski, VT, USA). Anti-C1q levels were calculated using a reference SLE sample [set as 1000 arbitrary units (AU)].

Flow cytometry

After 24 h of stimulation on different coatings described above, cells were collected for analysis by flow cytometry. To exclude dead cells, either DAPI (3 μM; Biolegend) or Fixable Viability Dye eFluor 780 (Thermo Fisher Scientific, Waltham, MA, USA) was used according to the manufacturer's instructions. To avoid unspecific binding of IgG, cells were incubated with 2 mg human IgG (Blood Transfusion Service SRC, Bern, Switzerland) mL^{-1} per 1 000 000 cells in FACS buffer [PBS supplemented with 1% (m/v) BSA and 1 mM sodium azide (both from Sigma-Aldrich)] at 4°C for 30 min. Additionally, appropriate biological and/or isotype controls were applied to ensure the specificity of the antibodies. Staining for surface marker expression was performed for 30 min at 4°C and included the following antibodies: mouse anti-human CD25 APC and CD69 PE (both from Immunotools) (antibody panel 1), or CD11c APC (Biolegend), CD40 FITC, CD80 FITC and CD86 FITC (all from BD) (antibody panel 2). After washing cells twice with FACS buffer, at least 20 000 events in the viable gate were acquired on a BD LSRFortessa (BD Biosciences) and analysed using FlowJo 10.7.1 to calculate mean fluorescence intensity (MFI).

For detection of intracellular TNF, IFN-γ and IL-10 in PBMCs after 24 h, brefeldin A (3 μg mL^{-1}; eBioscience, San Diego, CA, USA) was added for the final 4 h of cell culture. Following extracellular staining with CD4 BV510, CD8 BV711, CD14 PE/Cy7, CD19 BV421 and CD56 Alexa Fluor 488 (all from Biolegend) (antibody panel 3) for 30 min at 4°C, cells were fixed and permeabilised using Intracellular Fixation & Permeabilization Buffer Set (eBioscience) according to the manufacturer's instruction. Next, cells were incubated for 45 min at room temperature with mouse anti-human TNF PE, IFN-γ BV605 and rat anti-human IL-10 APC (all from Biolegend). A minimum of 100 000 events in the viable gate were acquired on a Cytek Aurora (Cytek Biosciences, Fremont, CA, USA).

Inhibition of intracellular TRAF6 and JAK3/STAT5 CD40 signalling

TRAF6-specific inhibitor 6877002 (IC_{50} 15.9 μM),[88] NF-κB-specific inhibitor JSH-23 (IC_{50} 7.1 μM)[89] and JAK3-specific inhibitor PF-06651600 (IC_{50} 33.1 nM)[90] were dissolved in DMSO and deionised water (all from Sigma-Aldrich), respectively, and sterile filtered using a 0.22 μm mixed cellulose ester membrane filter (Merck, Burlington, VT, USA). Final DMSO concentrations did not exceed 0.3% (v/v) in cell culture experiments.

Statistical analysis

The non-parametric statistical analyses between two groups were performed using the Wilcoxon signed-rank and Mann–Whitney *U*-test for paired and unpaired data; differences between multiple groups were determined using the Friedman test following the Tukey's multiple comparison test. Correlations were calculated using Spearman's rho. Statistical significance was considered with $^{*}P \leq 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Analyses

were conducted with GraphPad Prism 9.1.2 (GraphPad Software, San Diego, CA, USA).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Pascal Alexander Rabatscher: Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Marten Trendelenburg:** Conceptualization; formal analysis; funding acquisition; project administration; supervision; writing – original draft; writing – review and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Dryad Digital Repository at https://doi.org/10.5061/dryad.c866t1g7s.

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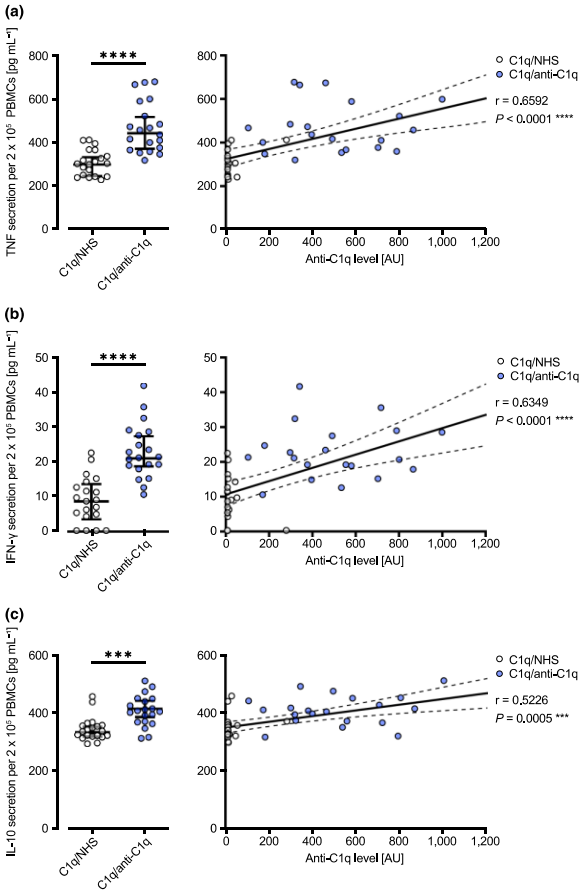
Supporting Information

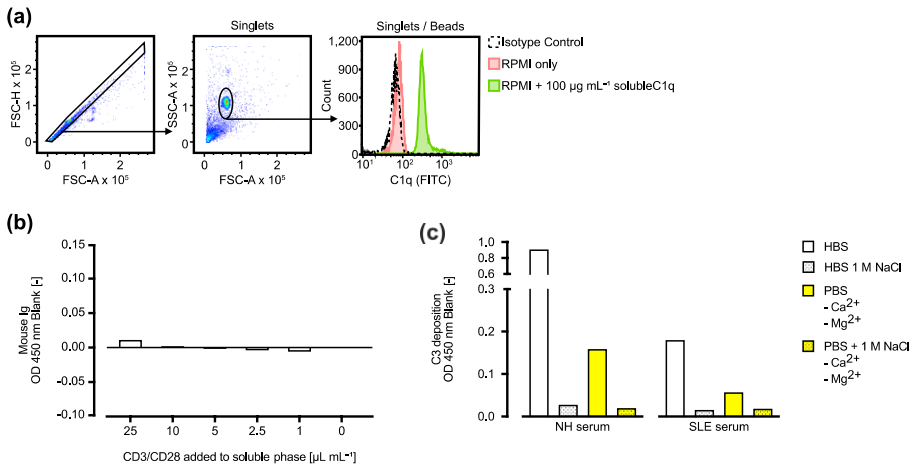
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Graphical Abstract

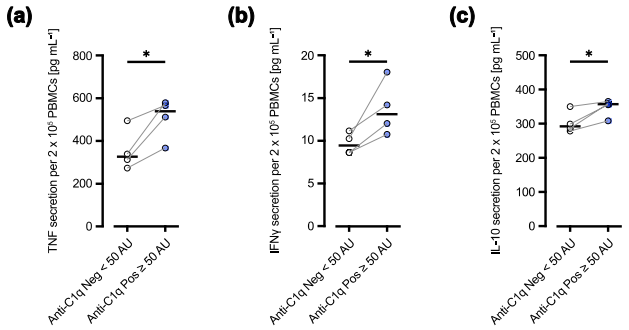
The contents of this page will be used as part of the graphical abstract of html only. It will not be published as part of main.



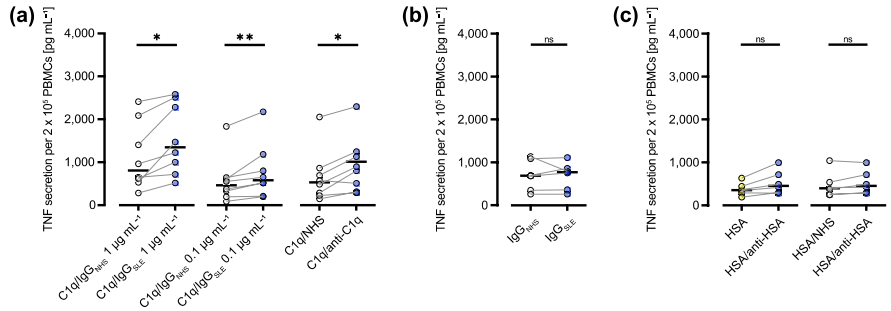
The findings of our study describe the immunological and cellular consequences of anti-C1q autoantibodies complexed with C1q on peripheral blood mononuclear cells (PBMCs). Whereas T cells alone were not directly affected in their activation and proliferation, we demonstrate that TNF, IFN-γ and IL-10 secretion in PBMCs is increased in the presence of C1q/anti-C1q complexes. Furthermore, the observed pro-inflammatory effect on monocytes is dependent on T cell activation and CD40–CD154 signalling.



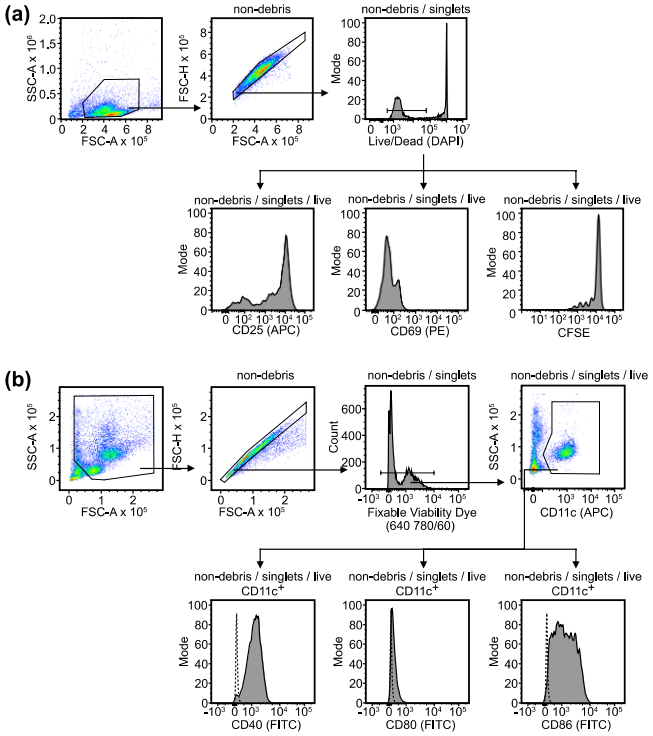
Supplementary figure 1 – C1q interacts with bound anti-CD3/CD28 T cell activating antibodies, whereas soluble tetrameric anti-CD3/CD28 antibody complexes do not. Additionally, complement activation was not observed with PBS 1 M NaCl buffer. (a) Dynabeads coupled to anti-CD3/CD28 antibodies (Life Technologies) were incubated in complete cell culture medium supplemented with or without 100 µg mL^{-1} soluble C1q at 37 °C. After 96 h, binding of C1q to Dynabeads was assessed by flow cytometry. **(b)** Similarly, binding of soluble tetrameric anti-CD3/CD28 complexes (Stemcell Technologies) to bound C1q was analyzed by an anti-mouse Ig ELISA. **(c)** Complement C3 deposition was measured by ELISA to determine complement activation in normal human (NH) and SLE patient (SLE) samples in the presence of bound C1q. Samples were diluted 1:100 in HEPES buffered saline supplemented with calcium and magnesium (HBS, pH 7.4), HBS 1 M NaCl, PBS lacking calcium and magnesium (pH 7.4), or PBS 1 M NaCl.



Supplementary figure 2 – Intra-patient comparison of anti-C1q negative and positive samples from different time points reveals increase in TNF, IFNγ, and IL-10. PBMCs activated by tetrameric anti-CD3/CD28 complexes were cultured on C1q preincubated with anti-C1q negative and positive sera each from the same patient ($n = 4$) but obtained at different time points at patient's history (i.e. intra-patient comparison) for 24 h. Cell culture supernatants were analyzed for **(a)** TNF, **(b)** IFNγ, and **(c)** IL-10 secretion by ELISA. Data points represent the median cytokine concentration obtained from PBMCs of four unrelated healthy donors from independent experiments exposed to one single serum sample. Horizontal lines represent median cytokine secretion. Connecting lines link serum samples of the same patient from different time points. Paired *t*-test, $*P < 0.05$.



Supplementary figure 3 – Increase in TNF secretion in PBMCs after T cell activation is specific to C1q/anti-C1q complexes. PBMCs activated by tetrameric anti-CD3/CD28 complexes were cultured on **(a)** NHS or SLE patient derived purified IgG complexed with C1q, or **(b)** coated NHS or SLE patient derived purified IgG (5 µg mL^{-1}) alone, or **(c)** HSA preincubated with anti-HSA positive normal human serum (HSA/anti-HSA), HSA preincubated with anti-HSA negative NHS (HSA/NHS), respectively. After 24 h, cell culture supernatants were analyzed for cytokine secretion by ELISA. Median cytokine concentrations of TNF are shown as horizontal lines. Data points represent independent experiments analyzing PBMCs from **(a)** eight and **(b and c)** six different healthy donors. Connecting lines link data points of a single donor used to obtain PBMCs. Wilcoxon matched-rank test, $*P < 0.05$, $**P < 0.01$, ns: not significant.



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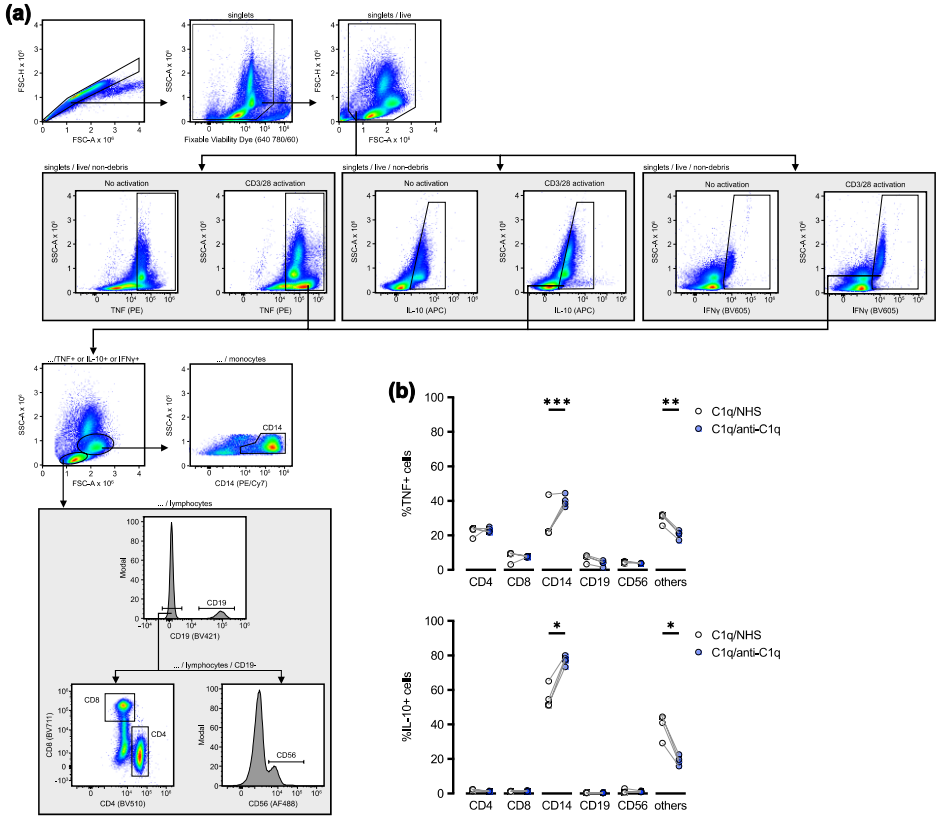
38 **Supplementary figure 4 – Flow cytometry gating strategy. (a)** Gating strategy for activation
39 markers and proliferation in isolated T cells after 24 and 96 h of stimulation with anti-
40 CD3/CD28 complexes, respectively. **(b)** $CD11c^+$ cell gating in PBMCs for expression levels
41 of co-stimulatory surface markers after 24 h of stimulation with anti-CD3/CD28 complexes.
42 Flow cytometry dot plots and histograms show one donor representative for six healthy donors.

4



Supplementary figure 5 – Detection of intracellular cytokines in PBMCs following T cell activation. PBMCs activated by tetrameric anti-CD3/CD28 complexes were cultured on C1q preincubated with anti-C1q positive SLE patient (C1q/anti-C1q) or anti-C1q negative healthy donor (C1q/NHS) derived serum for 24 h. Brefeldin A (3 µg mL^{-1}) was added for the final 4 h before cells were analyzed for intracellular TNF, IL-10, and IFNγ by flow cytometry. **(a)** Gating strategy used to obtain percentage of cells positive for TNF, IL-10 or IFNγ. Flow cytometry dot plots and histograms show one donor representative for four healthy donors. **(b)** Data are reported as percentage of TNF or IL-10 positive cells of four unrelated healthy donors used to obtain PBMCs. Two-way ANOVA test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Manuscript II

Epitope-Specific Anti-C1q Autoantibodies in Systemic Lupus Erythematosus

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Epitope-Specific Anti-C1q Autoantibodies in Systemic Lupus Erythematosus

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Objective: In patients with systemic lupus erythematosus (SLE) complement C1q is frequently targeted by autoantibodies (anti-C1q), that correlate best with active renal disease. Anti-C1q bind to largely unknown epitopes on the collagen-like region (CLR) of this highly functional molecule. Here we aimed at exploring the role of epitope-specific anti-C1q in SLE patients.

Methods: First, 22 sera of SLE patients, healthy controls and anti-C1q positive patients without SLE were screened for anti-C1q epitopes by a PEPperMAP® microarray, expressing CLR of C1q derived peptides with one amino acid (AA) shift in different lengths and conformations. Afterwards, samples of 378 SLE patients and 100 healthy blood donors were analyzed for antibodies against the identified epitopes by peptide-based ELISA. Relationships between peptide-specific autoantibodies and SLE disease manifestations were explored by logistic regression models.

Results: The epitope mapping showed increased IgG binding to three peptides of the C1q A- and three of the C1q B-chain. In subsequent peptide-based ELISAs, SLE sera showed significantly higher binding to two N-terminally located C1q A-chain peptides than controls ($p < 0.0001$), but not to the other peptides. While anti-C1q were associated with a broad spectrum of disease manifestations, some of the peptide-antibodies were associated with selected disease manifestations, and antibodies against the N-terminal C1q A-chain showed a stronger discrimination between SLE and controls than conventional anti-C1q.

Conclusion: In this large explorative study anti-C1q correlate with SLE overall disease activity. In contrast, peptide-antibodies are associated with specific aspects of the disease suggesting epitope-specific effects of anti-C1q in patients with SLE.

Keywords: autoantibody(ies), autoimmune diseases, complement, systemic lupus erythematosus (SLE), lupus nephritis

1 INTRODUCTION

Systemic lupus erythematosus (SLE) is the archetype of a systemic autoimmune disease. It is characterized by a dysregulated immune system, resulting in the generation of autoantibodies to numerous self-antigens and a broad spectrum of clinical manifestations. The exact cellular and molecular mechanisms leading to the disease remain incompletely understood (1) but may be elucidated by exploring the characteristics of self-antigens. One of these self-antigens is C1q, the first component of the classical complement activation pathway. Approximately 20-50% of unselected SLE patients have autoantibodies against C1q (anti-C1q) (2).

Positivity for anti-C1q is predictive for flares of lupus nephritis (LN) and anti-C1q levels correlate with overall disease activity (3). Additional lines of evidence suggest that these antibodies are directly involved in tissue injury (4): C1q deposition is a typical finding in severe LN and anti-C1q could be extracted from glomerular basement membrane fragments (5). Furthermore, C1q is a highly functional molecule (6) and experimental data support the assumption, that binding of anti-C1q alter those functions (7–10). However, the definite pathogenic role of the polyclonal anti-C1q remains to be determined and may strongly depend on the antibody binding site.

C1q is composed of 18 polypeptide chains (6 A-, 6 B- and 6 C-chains), that form six triple helices assembling to a structure that resembles a bouquet of tulips. Each chain has a short N-terminal region, followed by an ~81 residue-long collagen-like region (CLR) forming the stalk of the molecule and a ~135 residue-long C-terminal globular head region (gC1q) (11). The globular heads are mostly responsible for the recognition of target structures, e.g. Fc parts of bound immunoglobulins (12), surface proteins of pathogens and apoptotic cells (13). Upon binding of C1q, the CLR mediates immune effector mechanisms, including complement activation and enhancement of phagocytosis through interaction with cell surface receptors (14, 15).

Anti-C1q are polyclonal and primarily recognize neoepitopes on the CLR of C1q (16, 17) and to a lower extend also on gC1q (18, 19). These epitopes are cryptic, only exposed when C1q is in its bound form (20) and certainly located in different structures. However, so far little is known about the precise C1q epitopes (21, 22). As SLE patient-derived monoclonal anti-C1q Fabs recognize different C1q polypeptide chains in Western blot assay (22), they were used in a previous microarray-based peptide scan to identify peptide sequences recognized by anti-C1q (23). By this approach, Vanhecke et al. described a major linear epitope being located on the N-terminal C1q A-chain covering the arginine rich part of the chain, the so-called 'A08'. Interestingly, this region is also known as a major binding site for non-immunoglobulin molecules (24), and could even be an early epitope allowing cross reactivity of antibodies that primarily target EBNA-1 of Epstein Bar Virus (EBV) due to sequence homology (25). Epitope spreading might then lead to a more diverse antibody repertoire against the whole C1q molecule. As C1q has more functional subunits than just 'A08', e.g. the globular heads, the lysins in the C-terminal CLR that mediate the interaction of C1q with the $C1s_2C1r_2$ tetramer (26) and widely unknown regions, which are responsible for the interaction with C1q receptors (15), antibodies targeting these structures might have different functional consequences and thus mediate different disease manifestations.

The aim of this study was to explore epitopes of C1q and determine whether epitope-specific antibodies against C1q can be linked to specific disease manifestations.

Since the study by Vanhecke et al., was limited to the use of monoclonal antibodies, which do not mirror the polyclonal character of anti-C1q in patients, we used 22 SLE patient sera to determine the epitope landscape of C1q. In addition, we applied an advanced epitope mapping method based on densely overlapping linear as well as cyclized peptides, to increase sensitivity, since many epitopes rely on protein folding, which can hardly be detected with standard microarrays based on linear peptides alone (27). Subsequently we investigated the conspicuous peptide sequences by analyzing a large cohort of well-defined SLE patients provided by the Swiss Systemic Lupus Erythematosus Cohort Study (SSCS).

2 MATERIALS AND METHODS

2.1 Blood Samples

2.1.1 Cohort 1

For the epitope mapping, 22 serum or plasma samples were used from healthy blood donors, SLE patients being anti-C1q positive or negative respectively, and anti-C1q positive patients with diseases other than SLE. SLE patients fulfilled at least 4/11 American College of Rheumatology (ACR) revised criteria for the classification of SLE and were all recruited at the University Hospital Basel. Sera from healthy blood donors were obtained from the blood donation center in Basel.

2.1.2 Cohort 2

To determine the association of epitope-specific anti-C1q with disease manifestations, serum samples and related clinical data from 378 SLE patients were provided by the SSCS. SSCS is a prospective, nationwide, multicenter and longitudinal study of SLE patients living in Switzerland (28). SSCS includes adult SLE patients (> 17 years old) who fulfill at least 3/11 ACR revised criteria for the classification as SLE at the time of inclusion and who had given written informed consent. Patients were solely selected based on the availability of complete disease activity scores (SELENA-SLEDAI and PGA) and available plasma sample at the time of study visit.

Plasma samples of 100 healthy, sex matched blood donors from the blood donation center in Basel served as a reference.

2.2 Data Collection

Samples and data from SLE patients and healthy blood donors were collected cross-sectionally between October 2010 and June 2018. Laboratory parameters were assessed by the individual centers. SLE manifestations were defined using the ACR revised classification criteria (29, 30). Disease activity was assessed by the

Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score with the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) modification (31).

Additionally, we used a Physician's Global Assessment (PGA) score with a 4-point scale of disease activity, ranging from 0 (inactive) to 3 (very active). Both scores were used with a 30-day window (32). Active SLE disease was defined as a SELENA-SLEDAI ≥ 6 and PGA ≥ 1 at the time of sampling.

2.3 Epitope Mapping

Peptide microarrays were manufactured by PEPperPRINT (Heidelberg, Germany). The peptide sequence of the CLR of C1q was laser printed in an array format. Measurements were performed with cyclized peptides of 7, 10, and 13 amino acid (AA) length and with linear peptides of 15 AA length. The cyclic constrained peptides were linked at the C- and N-terminus by a thioether linkage and anchored to the microarray surface. The linear peptides were printed as stripes continually bound to the surface of the microarray. Both linear and conformational cyclic peptides were expressed with a 1-AA shift. Peptide microarrays were screened according to the manufacturer's protocol (33) with the following specifications: The secondary antibody was a goat anti-human IgG (Fc) DyLight680 and a mouse monoclonal anti-HA (12CA5) DyLight800 antibody was used as a control. Assays were performed with serum or plasma dilutions of 1:500. Arrays were scanned using a LI-COR Odyssey Imaging System and microarray image analyses were done with PepSlide® analyzer. The optical density (OD) was converted to a digital scale leading to values that ranged from 0 to 16,052 arbitrary fluorescence units (aFU) in our study. The magnitude of binding intensity of IgG to certain peptides is presented as color in a Heatmap, in which the highest value was limited to 1'000 aFU to facilitate comparisons between binding intensities of smaller amplitude.

2.4 C1q-Derived Peptides

Peptides used for peptide ELISA were synthesized with ≥ 95% purity by peptides & elephants (Hennigsdorf, Germany) and named according to the position of their first AA in the C1q molecule (11). Accordingly, the previously described 'A08' (23) was renamed 'A15' in this study. The difference in numbering is due to the two AA increments used previously, while the current study used one AA increment being identical with the AA position in the molecule. Peptides used for the experiments are summarized in **Table 1**. The peptides were diluted in Invitrogen™ UltraPuren™ DNase/RNase-Free distilled water and stored at -80°C until further use.

2.5 Peptide and anti-C1q ELISAs

Peptide and anti-C1q ELISAs were performed as published previously (23), with some modifications to improve the signal to noise ratio. The ELISAs were performed throughout with TBS and the peptide ELISAs were incubated at 33°C instead of 27°C. The incubation step of peptide coating was shortened to one hour. The optimal serum dilutions were found to be 1:50 for the anti-C1q ELISA, as well as for the peptide ELISAs of B-chain derived peptides. The optimal serum dilution for ELISAs of A-chain derived peptides was 1:100. Before diluting the samples to their final concentration, they were vortexed and then centrifuged for 30 minutes at 4°C and 14'000g.

Bound antibodies to peptides or C1q were detected by incubation for 45 minutes (peptides) or one hour (C1q) with alkaline phosphatase (AP)-conjugated goat anti-human Fc (gamma) antibody diluted 1:1'000 (peptide ELISAs) or 1:5'000 (anti-C1q ELISAs). For the peptide ELISAs, the signal obtained from incubating every single sample with diluting buffer instead of peptide was considered background, and this OD value was subtracted from the peptide-specific peptide.

For further analyses we standardized the experiments by expressing the data in units relative to the OD values obtained from a reference SLE serum (set as 1'000 relative Units, reIU), which was used to establish a standard curve. The reference serum showed high level of binding in the peptide ELISA and anti-C1q ELISA respectively and was included on every second plate. Calibration curves were fitted using a sigmoidal four-parameter logistic model. If the background of a peptide ELISA was higher than foreground, reIU were set to zero. If the background-adjusted values were higher than the upper limit of the standard curve, measurements were repeated in a 1:1'000 dilution and if necessary, in a 1:10'000 dilution, for the A-chain derived peptides and in a 1:500, and if necessary 1:5'000 dilution, for the B-chain derived peptides. For each serum, all peptide ELISAs were performed simultaneously.

2.6 Statistical Analysis

Statistical analyses and graphical presentations were conducted using R software version 4.0.2. and GraphPad Prism version 9.1.0. Univariate analyses were used to describe baseline characteristics. Data for continuous variables are presented as median with interquartile range (IQR). Categorical data are presented as frequency and percentage.

TABLE 1 | Nomenclature and structure of the studied peptides.

Previous name	New name	C1qchain	N-term	Sequence
	A09	A	Biotin	GKKGEAGRPGRRGRP
A08	A15	A	Biotin	GRPGRRGRPGLKG
	A86	A	Biotin	NIKDQPRPAFSAIRR
	B41	B	n/a	cyclo[K(biotin)AGDHGEF]
	B43	B	n/a	cyclo[K(biotin)DHGEFGE]
	B83	B	n/a	cyclo[K[biotin)GESGDY]

A15 and A09 both contain the 'A08' core sequence (marked gray) described previously (25). The B-chain derived peptides are brought into a cyclic conformation by an amide bond.

Non parametric-tests were used throughout, because of a lack of normal distribution in peptide- and anti-C1q ELISA. Correlations were analyzed by Spearman's correlation coefficient and differences in antibody titers were analyzed by a two-sided Mann-Whitney test. Statistical significance was considered as $^{*}p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ respectively. For the ELISA data, we set a cutoff corresponding to <10% positivity of the controls in all assays. Univariate logistic regression models were used to examine the relationship between positivity in ELISAs and manifestations of SLE, taking the serological measures as predictors and the presence of different disease features as dependent variables. In addition, we examined the association between positivity in peptide ELISAs and disease duration at the time of blood sampling, taking disease activity as a potential confounder into account. Since we performed an explorative study with no prespecified key hypothesis, type I error control was not implemented. Statistical tests are therefore used only for descriptive purposes. We expressed the results of the logistic regression analyses as odds ratios (OR) with associated 95% confidence intervals (95% CI). Intervals have not been adjusted for multiplicity. Subsequently we performed receiver operating characteristic (ROC) curves to compare the diagnostic performance of the peptide- and anti-C1q ELISAs with regard to specific outcomes. To compare the AUC of two ROC curves, DeLong test was used.

2.7 Compliance with Ethical Standards

This study was approved by all responsible local ethical committees and Swissethics (Ethical Committee of the Canton Vaud, Switzerland Ref. No. 2017-01434). All procedures performed in this study involving human participants were in accordance with the ethical standards of the research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

3 RESULTS

3.1 Patients Characteristics

3.1.1 Cohort 1

Demographic and baseline characteristics of patients used for epitope mapping are summarized in **Supplementary Table 1**. Patient SLE 4 had been described in a previous case report (34). Patients with anti-C1q but disease other than SLE had complement C2 deficiency (n= 2) (35), hypocomplementemic urticarial vasculitis (HUVS) (n= 1) (36) and essential cryoglobulinemia (n= 1) (37).

3.1.2 Cohort 2

A total of 378 patients, of previously selected 392 SLE patients, met the inclusion criteria. The flow diagram of eligible patients is shown in **Supplementary Figure 1**. Demographic and clinical characteristics are summarized in **Table 2**.

Of the 378 patients 324 (85.7%) were female and 54 (14.3%) were male. The median age at the time of blood sampling was 42 (32–54) years and the median SLE disease duration since diagnosis was 5 (1–13) years. At the time of the study visit 131/378 (34.7%) of the patients had active disease, defined as a PGA ≥ 1 and SELENA-SLEDAI ≥ 6 (31). The main clinical manifestations of the study population as defined by the SELENA-SLEDAI are shown in **Table 2**. The sex-matched control group consisted of 85 (85%) women and 15 (15%) men. Their median age at the time of blood sampling was 48 (38–60) years.

3.2 Epitope Mapping

In a first step we investigated the binding of IgG to peptides covering the CLR of the C1q A-, B- and C-chain, using peptides in different lengths and in a linear as well in a cyclic conformation. We initially tested 8 samples; four from anti-C1q positive SLE patients, two from anti-C1q negative SLE patients and two from healthy control donors.

Results are shown in **Figure 1**. Signal intensities from 10 and 13 AA cyclic peptides yielded similar results independent of the peptide lengths and were less strong than from 7 AA peptides. Considering the 7 AA cyclic peptides, a peak-signal was observed in peptides 13 to 19 of the A-chain, all containing the previously described 'A08' core sequence (25). The cyclic B-chain derived peptides showed two peaks at position 41 and 43, which were not present in the linear conformation. In the following these peptides are called B41 and B43. The C-chain derived peptides did not show consistent signal elevations. Within the 15 AA peptides in linear conformation, two constant signals appeared at position 9 and 86 of the C1q A-chain, hereafter referred to as A09 and A86 respectively. With regard to the B- and C-chains we did not observe patterns of binding intensities shared by several SLE patients to any of the 15-AA peptides in linear conformation.

In a second step we analyzed 16 additional serum samples, covering a broader disease spectrum, but limited the analysis to 7 AA cyclic peptides from the whole CLR of the C1q B-chain and the N-terminal part of the C1q A-chain covering the already described 'A08' epitope. Among all samples, 2 sera were investigated in both experiments and served as an internal control. Results are shown in **Supplementary Figure 2**. Binding intensities to peptides B41 and B43 were detected in all patient groups, but were slightly lower in healthy donors. Similarly, binding to peptide position 83 of the B-chain (B83) can be seen in all patient groups, although less pronounced in the control group.

3.3 Prevalence and Clinical Association of Autoantibodies

To characterize the clinical significance of the candidate epitopes which stand out in the epitope mapping, we established a peptide ELISA. Examined peptides are shown in **Table 1**. As an internal control, we measured antibodies against A15 (anti-A15; formerly called 'anti-A08') and anti-C1q as well. **Figure 2** shows the distribution of measured autoantibodies in SLE patients and controls as well as their correlation among each other. SLE IgG showed significantly higher binding to C1q, A15 and A09 and slightly lower binding to B41 when compared to controls. No

TABLE 2 | Demographic and clinical characteristics of patients with systemic lupus erythematosus and control group (normal blood donor).

	SLE group, n= 378[†]	Control group, n= 100
Female, n (%)	324 (85.7)	85 (85)
Male, n (%)	54 (14.3)	15 (15)
Disease Classification at time of inclusion		
American College of Rheumatology criteria, median (IQR)	5 (4–6)	
Ethnicity		
Caucasian, n (%)	280 (74.1)	
African, n (%)	38 (10.1)	
Asian, n (%)	37 (9.8)	
Native American, n (%)	18 (4.8)	
Other, n (%)	2 (0.5)	
Unknown (%)	3 (0.8)	
Age		
At blood sampling, median (IQR)	42 (32–54)	48 (38–60)
Disease duration since Diagnosis of SLE (IQR)	5 (1–13)	
Disease Activity and Clinical Features		
Active disease ‡, n (%)	131 (34.7)	
Fever, n (%)	24/377 (6.4)	
Arthritis, n (%)	84/375 (22.4)	
Active muco-cutaneous involvement §, n (%)	119/373 (31.9)	
Vasculitis, n (%)	8/377 (2.1)	
Serositis, n (%)	22/372 (5.9)	
CNS involvement ¶, n (%)	12/375 (3.2)	
Leukopenia, n (%)	53/372 (14.2)	
Thrombocytopenia, n (%)	31/373 (8.3)	
Proteinuria, n (%)	56/298 (18.8)	
Hematuria, n (%)	63/340 (18.5)	
Low Complement, n (%)	112/341 (32.8)	
Anti-ds-DNA antibodies, n (%)	167/340 (49.1)	
Anemia, n (%)	126/371(34.0)	
Elevated ESR #, n (%)	103/339 (30.4)	
Anti-Phospholipid antibodies, n (%)	59/183 (32.2)	

[†]n=378 unless otherwise stated, [‡]active disease was defined as SELENA- SLEDAI ≥ 6 and PGA ≥ 1, §Active muco-cutaneous involvement defined as malar rash or mucosal ulcers or alopecia at time point of blood sampling, ¶CNS involvement was defined as psychosis, seizure or organic brain syndrome at time of blood sampling, [#]ESR= erythrocyte sedimentation rate.

significant differences in IgG binding was observed for the other peptides.

Since anti-C1q autoantibodies are present in up to 10% of healthy individuals and in analogy to previous study data (38, 39), we chose a cutoff for positivity by accepting <10% positive healthy blood donors in all assays. With this cutoff, 65/378 (17%) of the SLE patients were anti-C1q positive, 159/378 (42%) anti-A09 positive, 123/378 (32.5%) anti-A15 positive, 34/378 (8.9%) anti-A86 positive, 32/378 (8.5%) anti-B41 positive, 53/378 (14%) anti-B43 positive and 29/378 (7.7%) anti-B83 positive. Antibodies directed against C1q-derived epitopes correlated weakly with antibodies against intact C1q (anti-C1q) (r= 0.1 - 0.2). Anti-A09 and anti-A15 showed a strong correlation with each other (r= 0.7), but both had only a moderate correlation with anti-A86 (r= 0.4). Auto-antibodies directed against B-chain derived epitopes showed strong correlations among each other (r= 0.6) but only weak correlations to A-chain derived epitopes (r= 0.1 – 0.3).

To explore the assumed relationship between measured autoantibodies and manifestations of SLE, univariate logistic regression was conducted, taking positivity in ELISAs as binary predictor and the presence of different disease features as binary dependent variable. **Figure 3** shows OR's and 95% CI's of SLE features as a function of positivity in anti-C1q- and A-chain derived peptide ELISAs. The corresponding values in numbers are shown in **Supplementary Table 2**.

While anti-C1q positivity correlated strongly with overall disease activity as well as with several SLE features, positivity for autoantibodies to A-chain derived peptides correlated only weakly with disease manifestations: Anti-A09 correlated with fever, arthritis, thrombocytopenia, and with the occurrence of anti-dsDNA antibodies. Patients with anti-A15 had an increased probability of having active disease, arthritis, leukopenia, low complement and antiphospholipid antibodies. Patients with anti-A86 were more likely to have vasculitis and leucopenia. Univariate logistic regression for anti-B-chain derived peptide-ELISAs showed solely a weak correlation between anti-B43 and arthritis (OR= 1.995, CI= 1.046 - 3.708) and anti-dsDNA antibodies (OR= 2.387, CI= 1.232 - 4.824) and are shown in **Supplementary Figure 3** and **Supplementary Table 3**.

Since it was previously described that anti-A15 (formerly called 'anti-A08') show a stronger correlation with SLE disease activity and nephritis than anti-C1q (10), we established additional ROC curves to allow a better interpretation of the diagnostic performance regarding those endpoints. ROC curves are shown in **Figure 4**. Regarding the discrimination between SLE patients and healthy donors, autoantibodies directed against the N-terminal part of the A-chain had a significantly larger AUC than anti-C1q (0.75 versus 0.64; $p < 0.01$). In contrast, the diagnostic performance of those autoantibodies taking the outcome active disease into account was not significantly

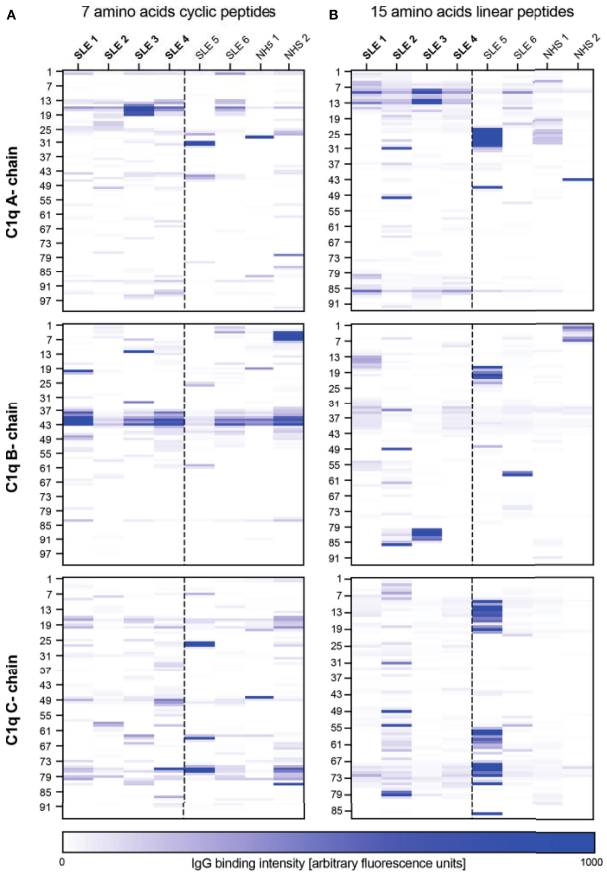

FIGURE 1 | Epitope mapping of the collagen-like region of C1q. Six patients with SLE and two healthy blood donors were screened for antibodies against peptides of the CLR of C1q (A-, B- and C-chain). The heatmap color represents the intensity of the antibody binding signal in each sample (column) to each peptide, named according to the position of their first AA on the C1q molecule (rows, left site). Patients in bold were anti-C1q positive at the time of blood collection, all others anti-C1q negative. **(A)** 7 AA peptides in cyclic confirmation. **(B)** 15 AA linear peptides.

different (AUC anti-A09: 0.59, AUC anti-A15: 0.61, AUC anti-C1q: 0.66; p= 0.09 for comparison of anti-A09 vs anti-C1q and p= 0.19 for anti-A15 vs anti-C1q). Regarding the occurrence of proteinuria, which is a typical finding in LN, anti-C1q show significantly better diagnostic performance than anti-A09 and anti-A15 respectively (AUC anti-A09: 0.49, AUC anti-A15: 0.55, AUC anti-C1q: 0.74; $p < 0.0001$ comparing anti-C1q versus anti-A09 and anti-A15 respectively).

To investigate the association between anti-C1q, -A09 and -A15 and disease duration we conducted a multivariate logistic regression, adjusting for disease activity. **Supplementary Figure 4** shows the graphical presentation of this multivariate regression using disease duration and activity as predictors and the possibility of positive autoantibodies as outcome. Adjusted Odds ratio for being autoantibody positive per one year of disease duration was 0.94 (CI's= 0.9 – 0.98) for anti-C1q, 0.98 (CI's= 0.95 - 1) for anti-A09 and 0.97 (CI's= 0.95 – 0.99) for anti-A15. The probability of having active disease was markedly higher when having positive anti-C1q (OR_{adj}= 4.86, CI's= 2.67-9.08), than anti-A09 (OR_{adj}= 1.43, CI's= 0.9 - 2.27) or anti-A15

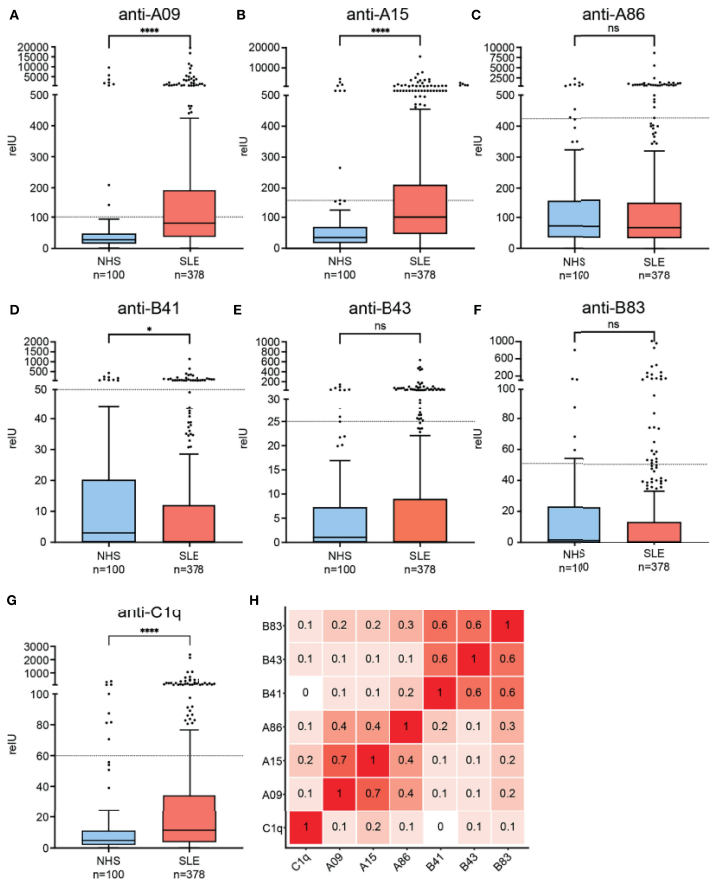

FIGURE 2 | Binding of IgG from SLE patients and healthy controls to candidate epitopes of the collagen-like region and correlation of autoantibodies among each other. **(A–G)** Graphs are named according to examined epitopes and show Tukey's boxplots with whisker lengths of 1.5x interquartile range. Outliers are shown as dots. Since the data are markedly skewed, Y-axis is segmented. Cutoffs for positivity are indicated by dashed lines. Statistical significance was considered as *p ≤ 0.05, ****p < 0.0001 respectively, ns, not significant. **(H)** Correlation-plot showing spearman correlation coefficients of measured autoantibodies among each other.

antibodies (OR_{adj}= 1.67, CI's= 1.03 – 2.68). There was no evidence of multicollinearity.

4 DISCUSSION

This study aimed at identifying and exploring the clinical relevance of epitope specific autoantibodies against complement C1q (anti-C1q) in patients with SLE. Considering the multiple functions of C1q, the role of C1q in SLE as well as the striking association of anti-C1q with active LN and SLE disease activity, a better understanding of epitopes of C1q could aid understanding the pathogenic mechanisms of SLE as well as improving diagnostic procedures. By using SLE patient sera in an advanced epitope mapping method, we first identified three epitopes on the C1q A- and three on the B-chain. In subsequent exploration of clinical relevance of these epitopes in a large cohort of patients by peptide-ELISA, two of the

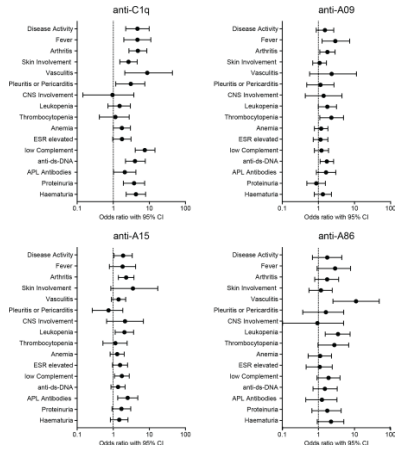

FIGURE 3 | Univariate Logistic Regression. Positivity in ELISAs as binary predictor and presence of disease manifestations as binary dependent variable. The graphs show odds-ratios and 95% confidence intervals of SLE manifestations. ESR, erythrocyte sedimentation rate; APL, antiphospholipid.

investigated peptides were significantly better recognized by serum IgG of SLE patients than of healthy controls. In addition, positivity for four of the investigated peptide-specific antibodies showed associations with selected SLE disease manifestations. These primarily explorative analyses might point to distinct functional properties of the measured peptide-specific antibodies.

The most obvious association with SLE was found for IgG antibodies targeting an epitope on the N-terminal C1q A-chain. The corresponding peptides were named 'A09' and 'A15' respectively, based on the position of their first AA on the CLR of C1q. Anti-A09 correlated with fever, arthritis, thrombocytopenia, and the occurrence of anti-dsDNA antibodies, while patients with anti-A15 had an increased probability of having active disease, arthritis, leukopenia, low complement and antiphospholipid antibodies. Regarding the occurrence of proteinuria, which is a typical finding in lupus nephritis, anti-C1q showed significantly better diagnostic performance than anti-A09 as well as anti-A15. This finding apparently is not in line with previous studies which showed that anti-A15-ELISA is more specific and more sensitive than a conventional anti-C1q assay for the detection of active lupus nephritis in SLE patients (10) (23). These differences in observation might be explained by the differences in patient selection and number. Both previous studies examined exclusively (10) or predominantly (23) lupus patients with renal biopsy-proven lupus nephritis, whereas most patients analyzed in the present study had long lasting, stable disease resembling an unselected clinical outpatient cohort of patients with closely monitored disease. Furthermore the sample sizes of the preceding studies were substantially smaller, than in the present study, namely n=61 (23) and n= 210 (10) versus n= 378 in our study presented here.

Nevertheless the present study is in line with the study from Vanhecke et al. showing that anti-A15 is better in discriminating asymptomatic donor sera from SLE patient sera than anti-C1q. In the present study, this discrimination was in the same range as the reported diagnostic performance of anti-dsDNA antibodies (40). Hence, anti-A-15 might serve as a diagnostic marker for SLE. However, to determine the real discriminatory power, it will be of importance to also investigate anti-A09 and -A15 in other inflammatory rheumatic diseases, and to perform a direct comparison with anti-dsDNA antibodies.

Furthermore, anti-C1q showed a weak correlation with anti-A09 or anti-A15 antibodies, respectively as also observed by Vanhecke et al. (23). In line with these findings, Wu et al. recently described that anti-A15 antibodies derived from 10 lupus nephritis patients bound to A15 but not to intact C1q (41). Regarding the potential functional consequences of anti-A09 and -A15, it should be noted that both peptides include a major binding site of C1q for non-immunoglobulin molecules (24). With regard to the interaction of A09 compared to A15 with binding partners other than anti-C1q, we hypothesized that the arginines being present in A09 as well will lead to very similar interactions as observed for A15. However, the differences in correlation with clinical parameters between anti-A09 and ant-A15 suggest that either the antibodies have a different potential to interfere with the known interactions of C1q with the described non-immunological molecules and/or receptors, or may point to differences in interaction between the two sites themselves with these binding partners.

The mentioned core sequence was previously described to allow crossreactivity between antibodies directed against EBNA-1 of EBV and C1q (25). In addition, Wu et al. could show that BALB/C mice, which were immunized with the A15 peptide, developed anti-C1q antibodies. They concluded that A15 is important for development of anti- C1q antibodies, but epitope spreading might then lead to a more diverse antibody repertoire against the whole C1q molecule. In line with this finding, generation of anti-A09 and -A15 in SLE patients seem to be an early event in the course of the disease. Additionally, data from multivariate regression suggest that anti-A09 and -A15 have higher stability over time with lower dependency on disease activity (**Supplementary Figure 4**). Taken together, these results support the hypothesis, that molecular mimicry is an early event in the pathogenesis of SLE, with the formation of anti-A15 antibodies being an intermediate step, but might also explain the weak correlation between anti-A15 and anti-C1q.

With regard to IgG antibodies against the other described peptide epitopes, no overall differences in antibody levels between SLE patients and control sera were observed. However, when judging on the significance of these antibodies, one has to keep in mind that quantitatively peptide-specific anti-C1q are only representing a small fraction of total anti-C1q, and

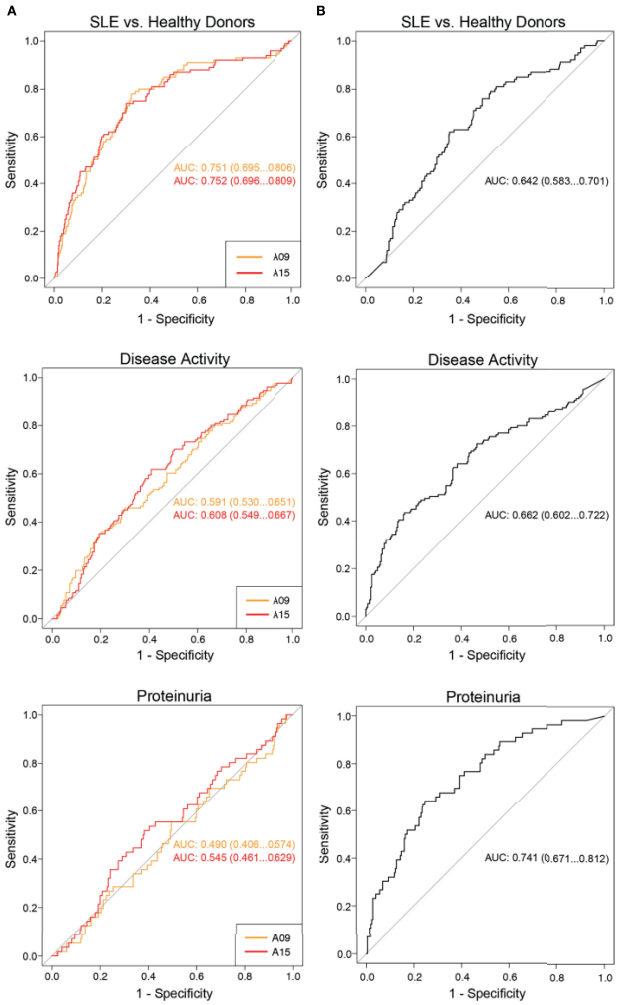

FIGURE 4 | Comparison of the diagnostic performance between anti-C1q and anti-A09/A15 as determined by ELISA. ROC curves analyzing the diagnostic performance of anti-A09, anti-A15 and anti-C1q regarding the discrimination of SLE patients from healthy donors, SLE patients with active versus inactive disease and proteinuria versus no proteinuria. **(A)** ROC curves of anti-A09 and anti-A15. **(B)** ROC curves of anti-C1q.

they only occur in a subgroup of patients that is likely to be too small to have an impact on overall differences between unselected SLE patients and healthy controls. As the study hypothesis was that antibodies against distinct epitopes of the multifunctional C1q molecule are associated with a specific disease expression, we thus also explored the association of the peptide antibody positivity with the clinical presentation of SLE. Patients with anti-A86 were more likely to have vasculitis and leucopenia, and the presence of anti-B43 was associated with arthritis and anti-dsDNA antibodies.

However, because of the explorative character of these analyses, confirmatory studies in large cohorts of SLE patients covering a broad spectrum of SLE manifestations and taking interrelations into account will be required to define the definite role of the described peptide antibodies.

Lastly, with regard to anti-C1q levels we observed that anti-C1q are associated with a much wider range of clinical disease manifestations than previously described. So far, anti-C1q antibodies have mainly been studied in association with LN (42–44). However, while confirming this known association in the present study, we also observed a clear association with arthritis OR= 4.811 (2.722 - 8. 543), skin involvement OR= 2.646 (1.522 -4.613), vasculitis OR= 8.757 (2.093 -43.629) and serositis OR= 3.065 (1.175 - 7.514). Therefore, anti-C1q could be more broadly considered as marker of SLE disease activity. This observation could be due to the large number of SLE patients investigated in our study. To the best of our knowledge, to date our study is the largest ever on anti-C1q in SLE patients.

The main limitation of the present study is its retrospective observational character. In addition, in spite of the large number of investigated patients, the sample size was still too small to make a clear statistical statement for some of the investigated disease features and thus would require even larger cohorts. Moreover, longitudinal data on the described antibodies will be of importance in the future. Lastly, despite the extensive character of our epitope mapping, the expression of peptides only partially resembles the conformation of the corresponding peptide sequences as part of the complete C1q molecule, and the expression of peptides probably differed between their expression in the initial epitope mapping versus the ELISAs performed in the large SLE cohort. Furthermore, our methodologies were not able to detect and describe antibodies against epitopes involving two or more chains of C1q. Thus, the described peptide-specific antibodies are likely still representing only a fraction of total anti-C1q.

In conclusion, in this exploratory and largest study to date on anti-C1q in SLE patients we describe six candidate epitopes of anti-C1q and their clinical associations in SLE patients. Two N-terminal located A-chain epitopes, which provide good discrimination between SLE patients and healthy individuals, might serve as a biomarker of the disease. In addition, peptide-specific anti-C1q were found to be associated with specific disease manifestations, but their potential impact on clinical patient management and for the understanding of pathogenic mechanisms needs to be confirmed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Swissethics (ethical committee of the Canton Vaud, Switzerland Ref. No. 2017-01434). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. JK had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design. JK, JW, MT. Acquisition of data. JK, JL, AK-G, CC, UH-D, CR, and MT. Analysis and interpretation of data. JK, PR, SV, and MT.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.761395/full#supplementary-material

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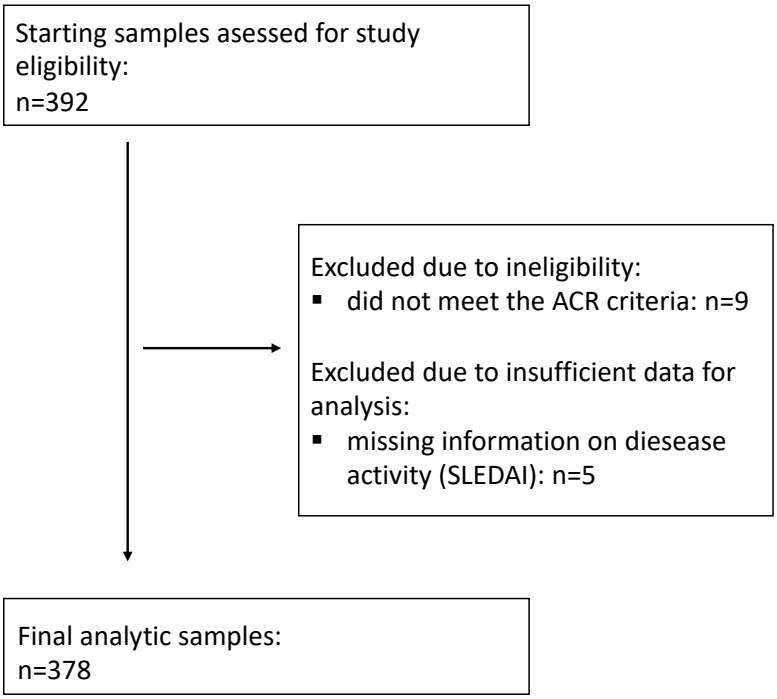
Supplementary Material

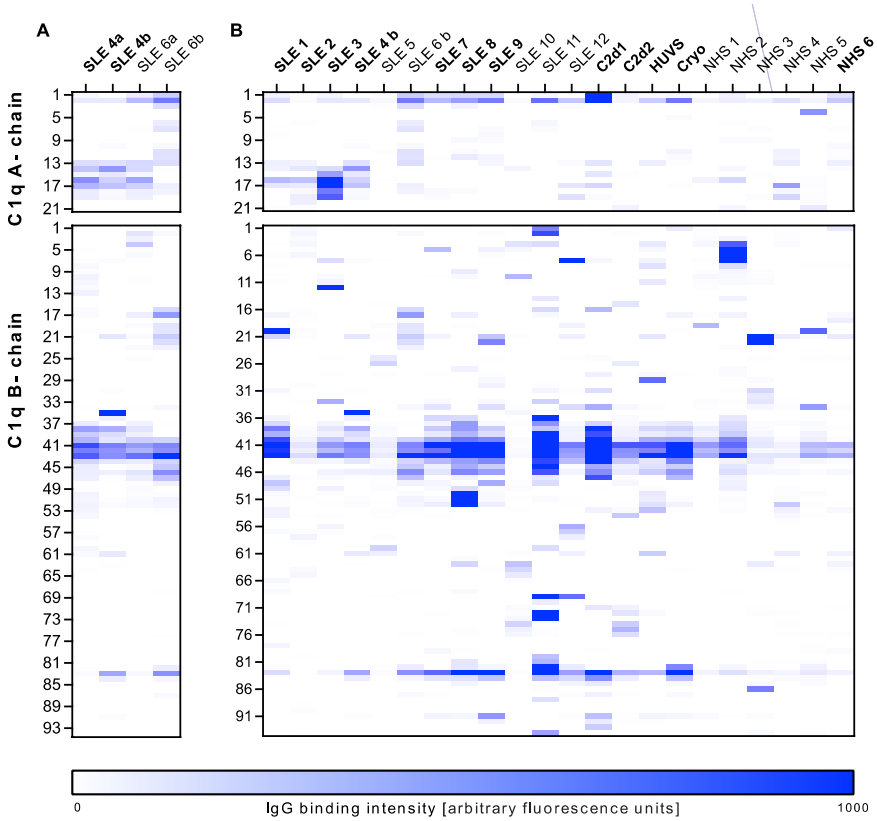
Supplementary Table 1. Baseline demographics and disease characteristics in patients used for epitope mapping

Sample	Dx.	Anti-C1q IgG	Clinical state	SLEDAI score	Age [y]	sex	Class of Lupus Nephritis (WHO)	Disease-Duration [y]
SLE 1	SLE	pos.	active	16	54	m	IV	0
SLE 2	SLE	pos.	active	17	52	f	IV	1,5
SLE 3	SLE	pos	active	10	24	f	III	0
SLE 4	SLE	pos.	active	10	32	f	IV	0
SLE 5	SLE	neg.	inactive	0	76	m	III	11
SLE 6	SLE	neg.	active	42	70	f	-	0
SLE 7	SLE	pos.	moderate	6	52	f	-	21
SLE 8	SLE	pos.	active	24	44	f	IV	4
SLE 9	SLE	pos.	active	38	49	m	IV	12
SLE 10	SLE	neg	inactive	6	65	f	III	13
SLE 11	SLE	neg.	inactive	3	62	f	IV	26
SLE 12	SLE	neg.	inactive	5	50	f	V	18
C2d1	other	pos.	active	-	57	m	-	9
C2d2	other	pos.	inactive	-	58	f	-	-
HUVS	other	pos.	active	-	59	f	IIa, Vb	unk.
Cryo	other	pos.	active	-	52	m	secondary MPGN type I	4
NHS 1	-	neg.	-	-	31	f	-	-
NHS 2	-	neg.	-	-	33	f	-	-
NHS 3	-	neg.	-	-	34	m	-	-
NHS 4	-	neg	-	-	unk.	unk.	-	-
NHS 5	-	neg.	-	-	unk.	unk.	-	-
NHS 6	-	pos.	-	-	28	f	-	-

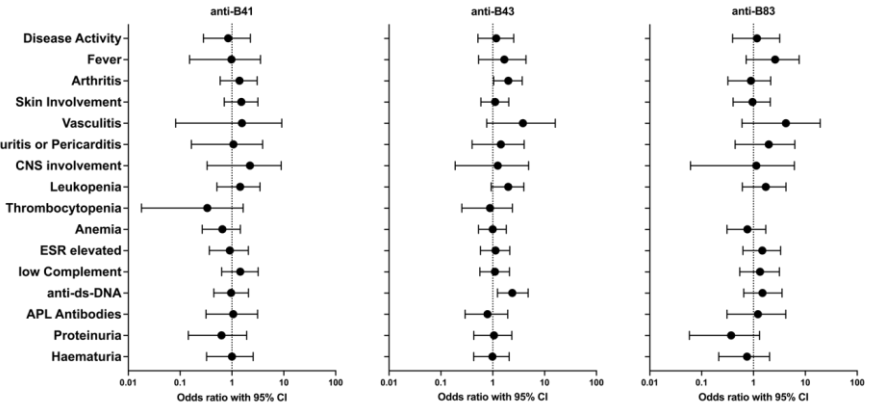
Characteristics of Patients included in the epitope-mapping. The clinical state was classified according to physician's global assessment index at the time of study visit. Anti-C1q-status was determined in the University Hospital Basel and by non-commercial anti-C1q ELISA. SLE = Systemic Lupus Erythematosus, NHS = Normal Human Serum, C2d1 and C2d2 = patients with complement C2 deficiency, HUVS = Hypocomplementic Urticaria Vasculitis Syndrome, Cryo = Patient with severe primary cryoglobulinemia, Dx= Diagnosis, SLEDAI = Systemic Lupus Erythematosus Disease Activity Index, ACR = American College of Rheumatology * assessment at time of sampling



Supplementary Figure 1. Flow chart of included and excluded patients.



Supplementary Figure 2. Epitope mapping of the CLR of C1q. Among the 16 samples used for the second epitope mapping, 2 were investigated in both experiments and served as controls (SLE 4 and 6). The appendix (a) refers to results obtained in experiment 1 and (b) for results obtained in experiment 2. Experiment 2 was limited to 7 amino acid cyclic peptides, derived from the whole CLR of the C1q B-chain and the N-terminal part of the C1q A-chain covering the 'A08' epitope (renamed A15). The heatmap color represents the intensity of the antibody binding in each sample (column) to each peptide, named according to the position of their first amino acid on the C1q molecule (rows, left). Patients in bold were anti-C1q positive at the time of blood collection, all others anti-C1q negative. (**A**) results of the binding intensities to peptides comparing binding intensities measured in the first versus the second experiment. (**B**) Pooled analyses of IgG binding to peptides in different patient groups**.**



Supplementary Figure 3. Univariate logistic regression with positivity in ELISAs as binary predictor and presence of disease features as binary dependent variable. The graphs show odds-ratios and 95% confidence intervals of SLE features. ESR= erythrocyte sedimentation rate. APL= antiphospholipid-antibodies. Odds ratio for thrombocytopenia in anti-B83 positive patients could not be calculated, since no anti-B83 positive patient had thrombocytopenia.

Supplementary Table 2. ORs and corresponding 95% CI's resulting from univariate logistic regression, using anti-C1q, anti-A09, anti-A15 and anti-A86 as predictor

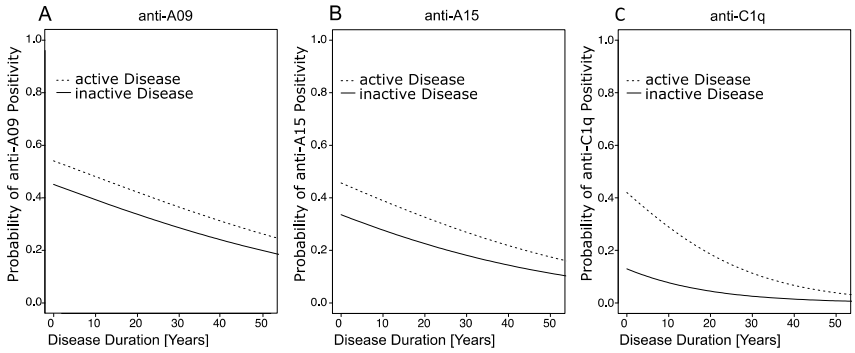
Disease Feature	anti-C1q	anti-A09	anti-A15	anti-A86
Disease Activity	4.596 (2.22 - 9.889)	1.528 (0.871 - 2.688)	1.905 (1.06 - 3.427)	1.77 (0.683 - 4.525)
Fever	4.685 (1.963 -11.026)	2.972 (1.272 - 7.501)	1.845 (0.787 - 4.253)	2.94 (0.922 - 7.95)
Arthritis	4.811 (2.722 - 8.543)	1.791 (1.1 - 2.931)	2.317 (1.406 - 3.82)	1.756 (0.791 - 3.695)
Skin Involvement	2.646 (1.522 -4.613)	1.084 (0.697 - 1.681)	1.424 (0.899 - 2.248)	1.183 (0.548 - 2.44)
Vasculitis	8.757 (2.093 -43.629)	2.353 (0.569 - 11.609)	3.95 (0.866 – 17.738)	11.3 (2.559 – 50.014)
Pleuritis or Pericarditis	3.065 (1.175 - 7.514)	1.151 (0.474 - 2.738)	0.747 (0.262 - 1.869)	1.625 (0.367 - 5.112)
CNS involvement	0.952 (0.144 - 3.725)	1.404 (0.432 - 4.568)	2.157 (0.661 -7.032)	0.94 (0.051 - 5.073)
Leukopenia	1.503 (0.714 - 2.979)	1.78 (0.993 - 3.217)	2.074 (1.145 - 3.745)	3.536 (1.554 - 7.69)
Thrombocytopenia	1.151 (0.413 - 2.762)	2.312 (1.1 -5.044)	1.16 (0.52 - 2.464)	2.8 (0.974 - 7.05)
Anemia	1.736 (1.002 - 2.991)	1.198 (0.776 - 1.848)	1.303 (0.826 - 2.048)	1.123 (0.519 - 2.33)
ESR† elevated	1.745 (0.968 - 3.108)	1.152 (0.719 -1.838)	1.568 (0.964 - 2.541)	1.094 (0.456 - 2.445)
low Complement	7.482 (4.072 - 14.297)	1.222 (0.775 - 1.927)	1.759 (1.094 - 2.824)	1.928 (0.916 - 4.028)
anti-ds-DNA	4.004 (2.181 - 7.733)	1.724 (1.12 - 2.664)	1.377 (0.875 - 2.172)	1.521 (0.708 - 3.364)
APL‡ - Antibodies	2.086 (1.021 - 4.246)	1.639 (0.879 - 3.073)	2.559 (1.345 -4.906)	1.256 (0.445 - 3.312)
Proteinuria	3.772 (1.904 - 7.4)	0.873 (0.477- 1.572)	1.713 (0.932 - 3.112)	1.778 (0.661 - 4.328)
Hematuria	4.255 (2.28 - 7.911)	1.339 (0.771 - 2.322)	1.486 (0.837 - 2.606)	2.263 (0.931 - 5.152)

ESR = erythrocyte sedimentation rate. APL = antiphospholipid. Odds ratio for thrombocytopenia in anti-B83 positive patients could not be calculated since no anti-B83 positive patient had thrombocytopenia.

Supplementary Table 3. ORs and corresponding CI's resulting from univariate logistic regression, using anti-B41, anti-B43 and anti-B83 as predictor

Disease Feature	anti-B41	anti-B43	anti-B83
Disease Activity	0.845 (0.281 - 2.267)	1.168 (0.517 - 2.544)	1.166 (0.395 - 3.2)
Fever	0.979 (0.152 - 3.549)	1.672 (0.535 - 4.387)	2.624 (0.722 - 7.602)
Arthritis	1.398 (0.591- 3.057)	1.995 (1.046 - 3.708)	0.896 (0.322 - 2.15)
Skin Involvement	1.517 (0.708- 3.161)	1.115 (0.592 - 2.041)	0.957 (0.403 - 2.113)
Vasculitis	1.558 (0.082 - 9.159)	3.828 (0.766 - 16.094)	4.222 (0.599 - 19.365)
Pleuritis or Pericarditis	1.067 (0.165 - 3.901)	1.433 (0.401 - 4.042)	1.968 (0.442 - 6.267)
CNS involvement	2.22 (0.331 - 8.924)	1.252 (0.189 - 4.928)	1.131 (0.061 - 6.154)
Leukopenia	1.439 (0.514 - 3.474)	1.985 (0.932 - 3.996)	1.723 (0.609 - 4.237)
Thrombocytopenia	0.334 (0.018 - 1.648)	0.886 (0.254 -2.389)	
Anemia	0.654 (0.267 - 1.452)	1 (0.532 -1.827)	0.763 (0.308 - 1.724)
ESR elevated	0.909 (0.366 - 2.067)	1.131 (0.58 - 2.134)	1.478 (0.627 - 3.336)
low Complement	1.45 (0.633 - 3.211)	1.106 (0.564 - 2.101)	1.342 (0.543 - 3.162)
anti-ds-DNA	0.964 (0.446 - 2.073)	2.387 (1.232 - 4.824)	1.492 (0.648 - 3.555)
APL- Antibodies	1.056 (0.316- 3.125)	0.793 (0.293 - 1.945)	1.216 (0.308 - 4.2)
Proteinuria	0.628 (0.144 - 1.919)	1.056 (0.431 - 2.331)	0.37 (0.058 - 1.31)
Hematuria	0.999 (0.324 - 2.558)	0.987 (0.428 - 2.076)	0.749 (0.214 - 2.037)

ESR = erythrocyte sedimentation rate. APL = antiphospholipid. Odds ratio for thrombocytopenia in anti-B83 positive patients could not be calculated since no anti-B83 positive patient had thrombocytopenia.



Supplementary Figure 4. Graphical presentation of multivariate regression taking disease duration (diagnosis - blood sampling) and -activity as predictors and possibility of positive autoantibodies as outcome. Two activity stratified lines show the probability of having autoantibodies over the years of disease duration. Adjusted odds ratios for being autoantibody positive per one-year disease duration were (**A**) 0.98 (CI's= 0.95 - 1) (**B**) 0.97 (CI's= 0.95 – 0.99) and (**C**) 0.94 (CI's= 0.9 – 0.98). Adjusted odds ratio for being autoantibody positive in case of active SLE disease were (**A**)1.43 (CI's= 0.9 - 2.27), (**B**) 1.67 (CI's = 1.03 – 2.68) and (**C**) 4.86, (CI's= 2.67- 9.08).