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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

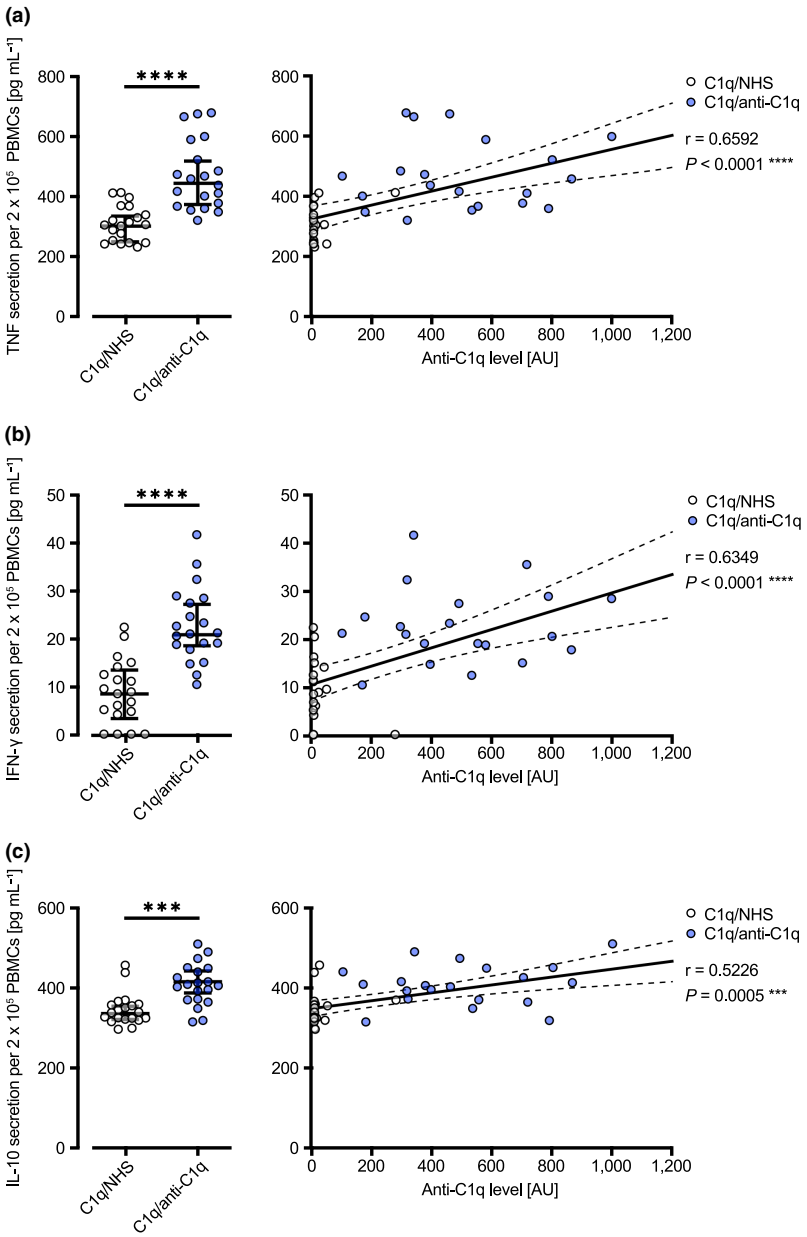

Figure 1. Increase in TNF, IFN-γ and IL-10 secretion in peripheral blood mononuclear cells (PBMCs) after T cell activation correlates with anti-C1q levels. PBMCs activated by tetrameric anti-CD3/CD28 complexes were cultured on C1q preincubated with the serum of 20 systemic lupus erythematosus patients (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.

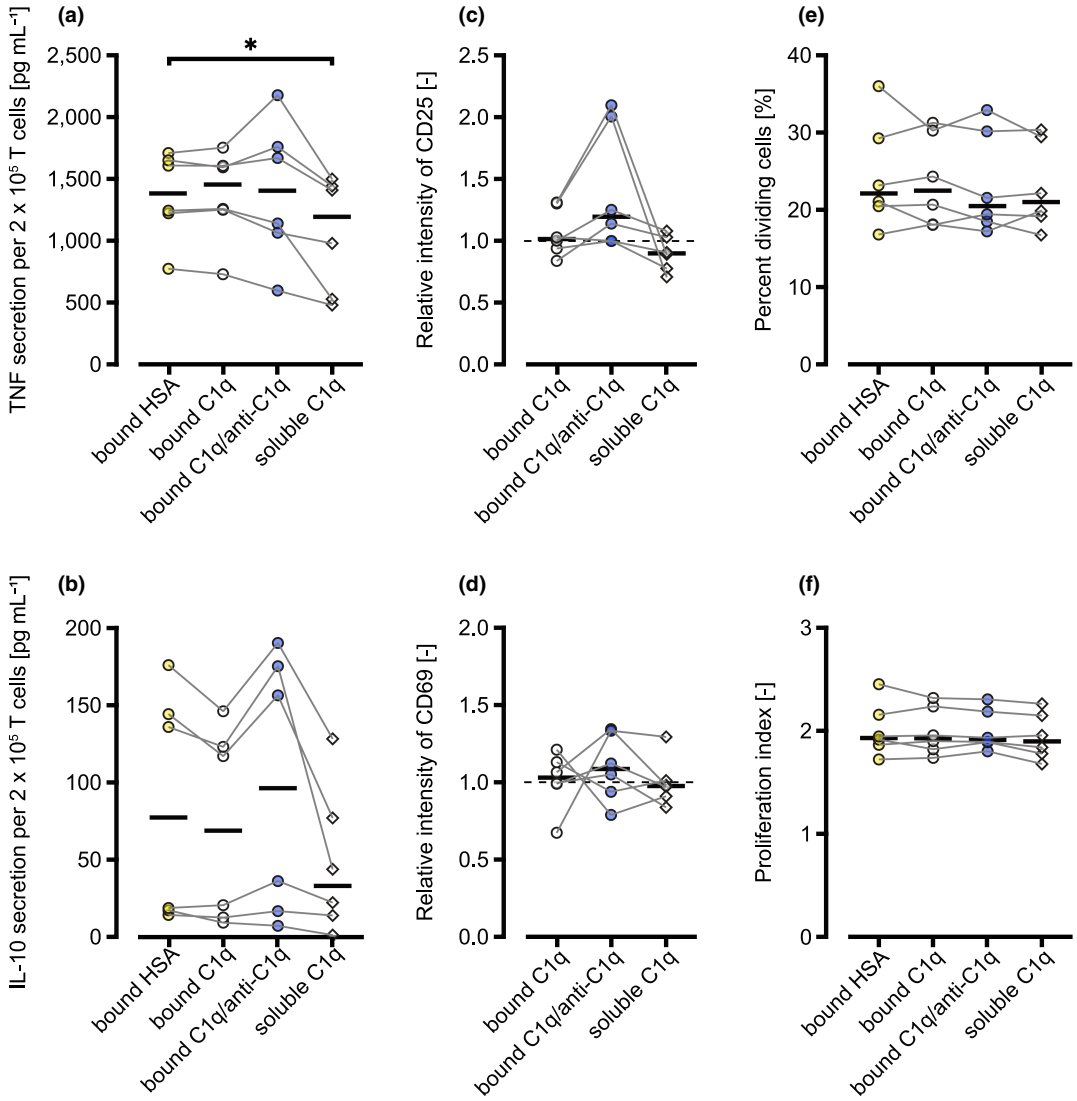

Figure 2. T cell proliferation, activation and IL-10 secretion in activated T cells are not affected by bound C1q, bound C1q/anti-C1q and soluble C1q, respectively, whereas TNF secretion is decreased in the presence of soluble C1q. T cells were isolated from peripheral blood mononuclear cells (PBMCs) 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

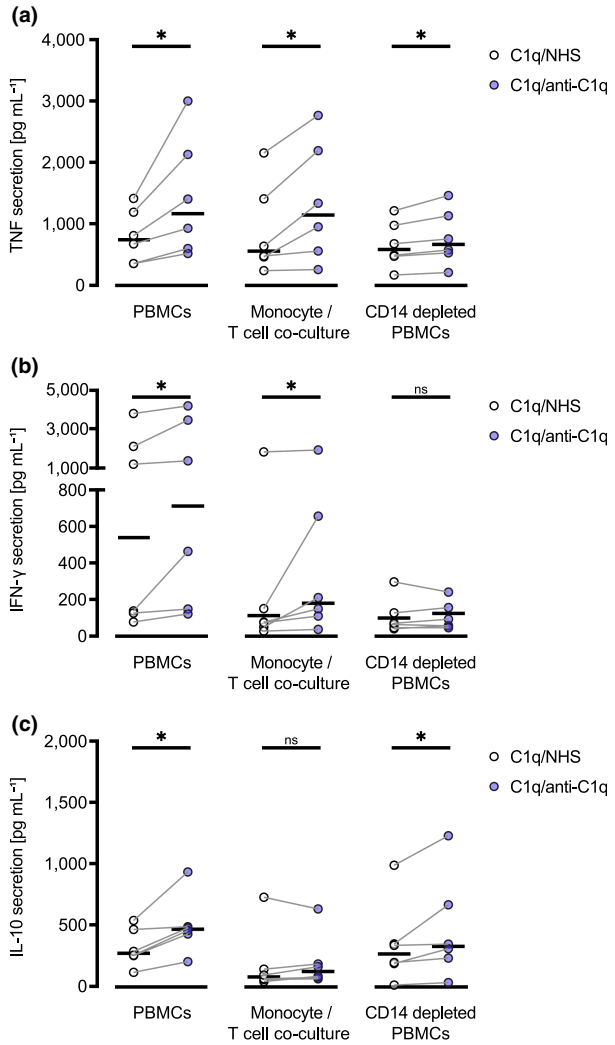

Figure 3. Presence of $CD14^+$ cells is essential for the increased cytokine secretion in the presence of C1q/anti-C1q complexes. Peripheral blood mononuclear cells (PBMCs), monocytes and T cells (co-culture 1:5 ratio) and CD14-depleted PBMCs (89–95% efficacy) were cultured on C1q preincubated with anti-C1q-negative 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

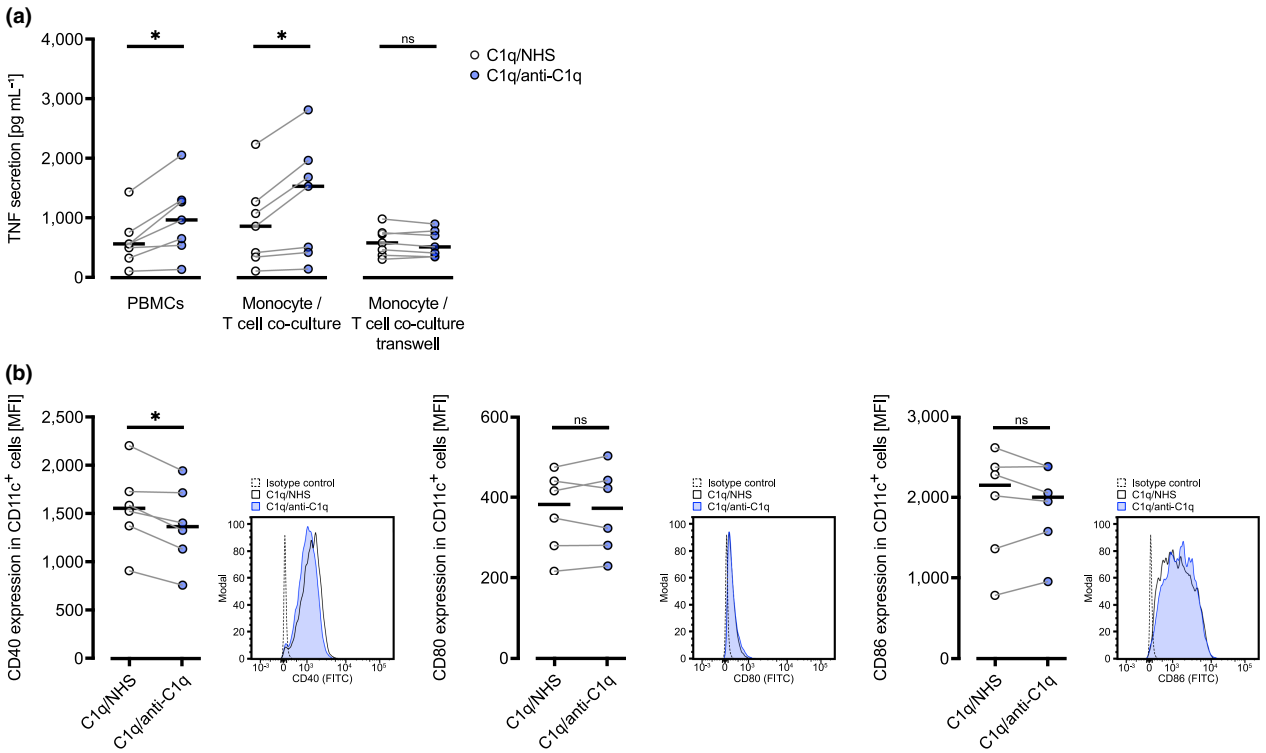

Figure 4. Increase in TNF secretion after exposure to C1q/anti-C1q complexes requires cell–cell contact between monocytes and T cells and involves CD40 downregulation in CD11^{+} cells. **(a)** Peripheral blood mononuclear cells (PBMCs) or monocytes and T cells (1:5 ratio) co-cultured either together 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

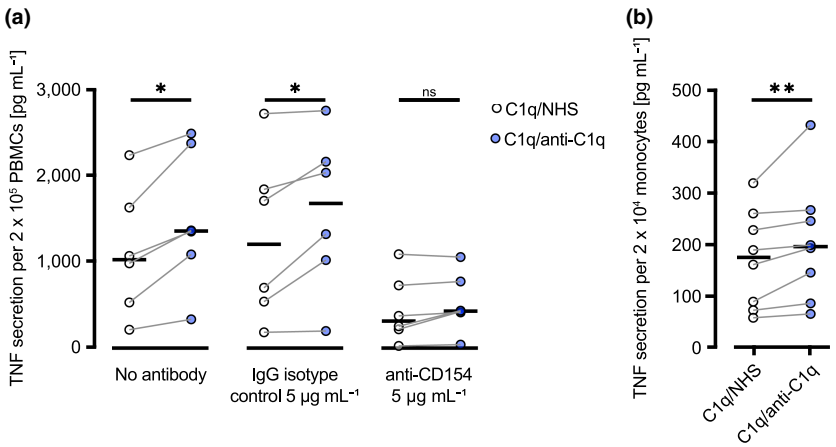

Figure 5. CD40 signalling to monocytes is essential for increased TNF secretion after exposure to C1q/anti-C1q complexes. **(a)** Peripheral blood mononuclear cells (PBMCs) from healthy donors were cultured in the presence of C1q, which was preincubated with anti-C1q-negative NHS (C1q/NHS) or anti-C1q-positive 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

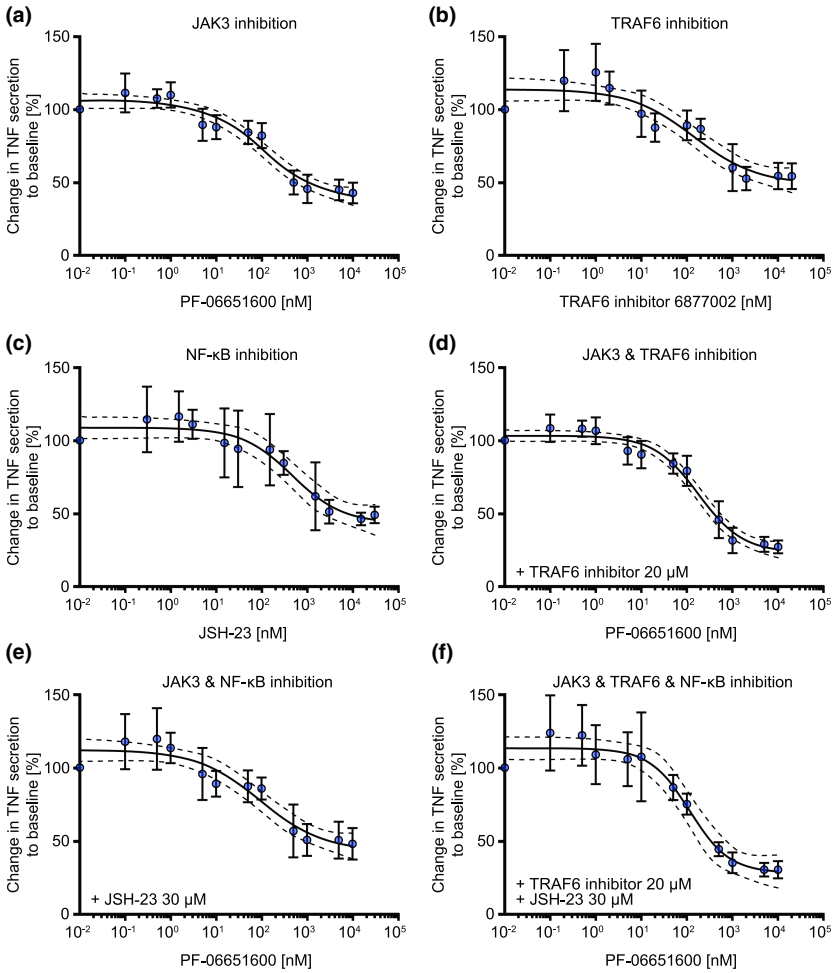

Figure 6. TNF secretion occurs via partially redundant intracellular CD40 signalling pathways JAK3-STAT5 and TRAF6. Monocytes were isolated from Peripheral blood mononuclear cells (PBMCs) of healthy donors and cultured in the presence of C1q/anti-C1q. Cells were preincubated with 500 U mL^{-1} IFN-γ 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

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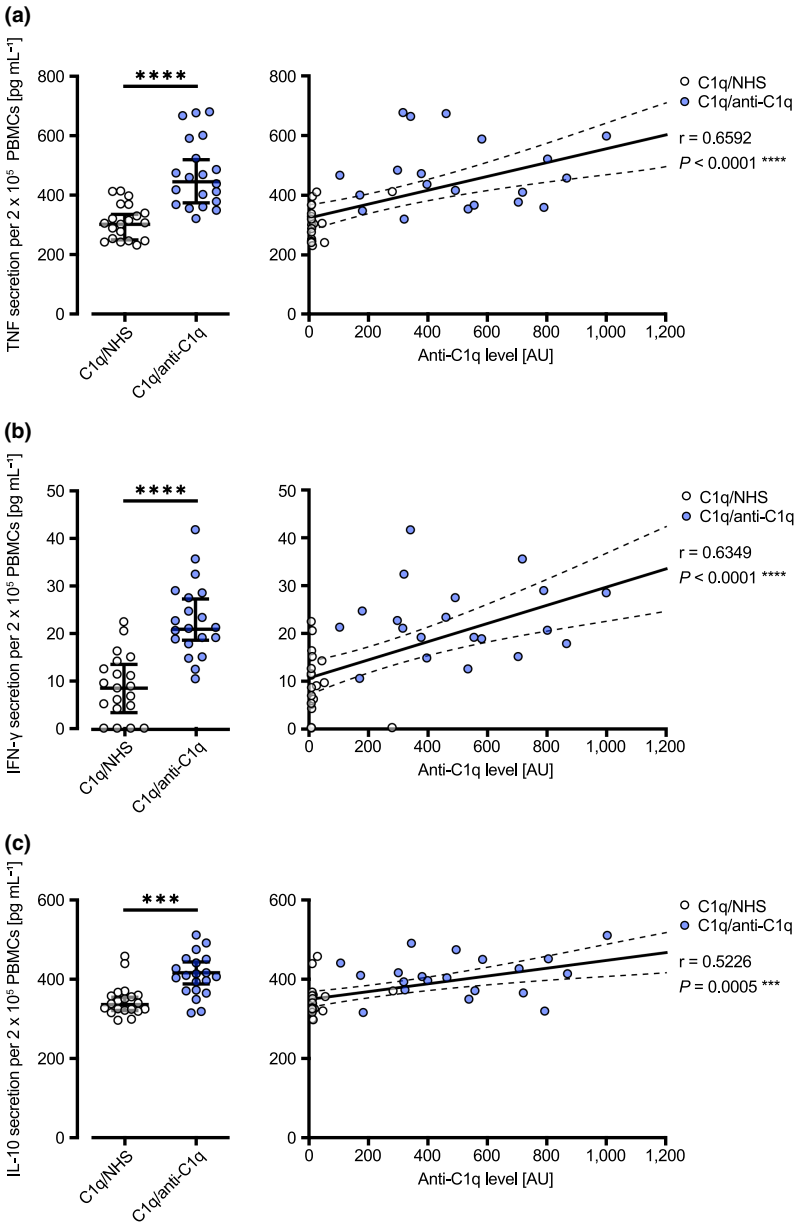

The findings of our study describe the immunological and cellular consequences of anti-C1q autoantibodies complexed with C1q on peripheral blood mononuclear cells (PBMCs). Whereas T cells alone were not directly affected in their activation and proliferation, we demonstrate that TNF, IFN-γ and IL-10 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.