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The Classical Complement Pathway in Transplantation: Unanticipated Protective Effects of C1q and Role in Inductive Antibody Therapy

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Though complement (C) deposition within the transplant is associated with allograft rejection, the pathways employed have not been established. In addition, evidence suggests that C-mediated cytolysis may be necessary for the tolerance-inducing activities of mAb therapies. Hence, we assessed the role of the classical C pathway in acute allograft rejection and its requirement for experimental mAb therapies. C1q-deficient (C1q-/-) recipients rejected allografts at a faster rate than wild-type (WT) recipients. This rejection was associated with exacerbated graft pathology but not with enhanced T-cell responses in C1q-/- recipients. However, the humoral response to donor alloantigens was accelerated in C1q-/- mice, as an early IgG response and IgG deposition within the graft were observed. Furthermore, deposition of C3d, but not C4d was observed in grafts isolated from C1q-/- recipients. To assess the role of the classical C pathway in inductive mAb therapies, C1q-/- recipients were treated with anti-CD4 or anti-CD40L mAb. The protective effects of anti-CD4 mAb were reduced in C1q-/- recipients, however, this effect did not correlate with ineffective depletion of CD4+ cells. In contrast, the protective effects of anti-CD40L mAb were less compromised in C1q-/- recipients. Hence, this study reveals unanticipated roles for C1q in the rejection process.

Key words: Antibodies, complement, transplantation

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This article is dedicated to Dr. Ernst J. Eichwald, who passed away on December 23, 2007.

Introduction

C1q initiates activation of the classical pathway of complement (C) and is considered a link between the innate and adaptive defense systems. The most well-understood function of C1q is its binding to the Fc portion of antigen-bound antibody that triggers the activation of the classical C pathway and subsequent formation of the membrane attack complex (MAC) (1). However, C1q has additional immune functions. T cells (2), dendritic cells (DC) (3), platelets and endothelial cells also express the C1q receptor (C1qR) (4). Binding of C1q to human endothelial cells up-regulates adhesion molecules and stimulates production of chemoattractants, resulting in migration of inflammatory cells (5,6). In addition, C1q binding to B cells stimulates antibody secretion (7,8). Paradoxically, interactions of C1q with its receptor on human T cells may reduce T-cell proliferation (2,9). Development of lupus has been associated with C1q deficiency in humans (10,11) and mice (12,13). Autoimmunity in C1q-/- mice is characterized by glomerulonephritis and the presence of apoptotic bodies and IgG deposits (14,15). Indeed, C1q binds to apoptotic bodies (16–18) and macrophages isolated from C1q-/- mice have a reduced capacity to phagocytize apoptotic cells (12). These observations highlight the importance of C1q in the clearance of inflammatory mediators. Hence, C1q has multiple roles in both inflammation and regulation of immune responses.

Activation of C has been associated with deleterious outcomes in transplantation. For example, C3, C3d and C4d are deposited in organs during rejection episodes (19,20) and the presence of the membrane-bound split products C3d or C4d has been associated with antibody-mediated organ rejection (21–26). C6 production by lung macrophages and subsequent MAC formation contribute to vascular injury in lung allografts (27). Additionally, C6 may contribute to cardiac allograft rejection (28), in that cardiac allograft survival is prolonged in C6-deficient recipient rats (28–30). Finally, C is activated following ischemia/reperfusion (I/R) injury (31–39) and may contribute to graft inflammation and subsequent rejection. These collective observations indicate that C proteins down stream from C1q are deleterious in the context of transplantation.

C1q and the classical C pathway may be required for the function of therapeutic mAb. For example, the clinically used mAb rituximab (anti-CD20) is thought to deplete

target cells by C-dependent cellular cytotoxicity, and the beneficial effects of rituximab in a mouse lymphoma model are dependent upon the presence of C1q (40). While the experimental mAb MR1 (anti-CD40L) has been thought to block receptor–ligand interactions, reports indicate that the effectiveness of this mAb may be dependent in part upon C (41,42). Therefore, further investigation of the role of C1q in the protective activities of mAb in transplantation is warranted. Herein, we report a previously unknown protective effect for C1q in allograft rejection and further define the role of C1q in suppressive inductive mAb therapies.

Materials and Methods

Mice

Breeder pairs of C1q-/- mice on a C57BL/6 (H-2^b) background (*C1qa-/-*) (14) were kindly provided by Marina Botto (Imperial College, UK) and were bred at Charles Rivers Laboratories (Wilmington, MA). Female C1q-/- mice were used in this study. Wild-type (WT) female C57BL/6 and BALB/c (H-2^d) mice were purchased from The Charles River Laboratories (Raleigh, NC). Relative to WT mice, C1q-/- mice have slightly elevated levels of C3 and decreased levels of C4 and MBL-A (43). The animals were kept under microisolator conditions following institutional guidelines reviewed and approved by the University of Michigan's Committee 'On The Use And Care Of Animals'.

Vascularized cardiac transplantation

C1q-/- and WT mice were transplanted with BALB/c heterotopic cardiac allografts as described (44). Transplant function was monitored by abdominal palpation.

Histology

Transplanted hearts were excised, fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H & E) to assess the nature and intensity of graft infiltration. Myocyte viability was determined by the presence of myocyte nuclei and cross-striation.

Isolation of graft-infiltrating cells (GIC)

Groups of three transplanted hearts were removed, pooled, minced and digested with 1 mg/mL collagenase A (Roche, Indianapolis, IN) for 30 min at 37°C. Tissue debris was allowed to settle at 1 x *g*, and the suspension containing GIC was harvested by pipette. RBCs were lysed by hypotonic shock and GIC were passed through a 30 μm pore size nylon mesh. For differential enumeration, GIC were placed on slides with a cytocentrifuge and stained with Wright's stain.

Flow cytometry

Splenocytes were isolated by mechanical dissociation followed by lysis via hypotonic shock and blocked in PBS containing 0.1% BSA, 0.025% NaN_3 and 10% FBS. After washing, 1×10^6 cells were stained with fluorochrome-conjugated anti-mouse CD4, CD19 and CD8 (all from BD Biosciences, San Jose, CA). Three-color flow cytometry was performed with a FACS Calibur (BD Biosciences) equipped with Cell Quest software.

ELISPOT assay for donor-reactive cytokine-producing cells

ELISPOT assays quantified primed, donor-alloantigen-reactive IFNγ (Th1) and IL-4 (Th2)-producing cells (45). Irradiated (1000 rads) donor splenocytes (4×10^5) were added to each well followed by 1×10^6 recipient splenocytes. After an 18-h incubation, plates were developed as described (45) and spots were quantified with an Immunospot Series 1 ELISPOT analyzer (Cellular Technology Ltd., Cleveland, OH).

Alloantibody assay

Donor-reactive alloantibodies were quantified as previously described (46,47). Briefly, 10^6 P815 (H-2^d) cells (American Type Culture Collection, Manassas, VA) were incubated with indicated dilutions of sera followed by FITC-conjugated rabbit anti-mouse IgG antibody (Zymed, San Francisco, CA). Unfixed samples were analyzed by flow cytometry. Data are reported as the mean channel fluorescence.

Immunohistochemistry

To detect IgG deposition within the graft, frozen sections of grafts were fixed in cold acetone and incubated with 1:150 dilution of goat anti-mouse IgG-HRP (Southern Biotech, Birmingham, AL) followed by AEC staining. To detect C3d and C4d deposition, sections of paraffin embedded tissue were fixed in methanol. A 1:500 dilution of goat anti-mouse C3d (R&D Systems, Minneapolis, MN) was added followed by secondary detection antibodies added as per R&D Systems' anti-goat cell and tissue staining kit. Slides were stained with rabbit anti-mouse C4d (Kindly provided by Dr. William Baldwin, Johns Hopkins (48)) at a 1:500 dilution, followed by detection and DAB development using the SuperPicTure™ Polymer Detection Kit, Zymed). Specificity of staining was ensured by staining of native hearts.

Inductive mAb therapy

To transiently deplete CD4+ cells, allograft recipients were injected i.p. with 1 mg of anti-CD4 mAb (hybridoma GK1.5, obtained from ATCC) on days −1, 0 and 7 relative to transplantation (49–52). For inductive anti-CD40L therapy, allograft recipients were injected i.p. with 1 mg of anti-CD40L (hybridoma MR1, kindly provided by Dr. Randy Noelle, Dartmouth Hitchcock Medical Center, Lebanon, NH) on days 0, 1 and 2 relative to transplantation (51,53).

Assessment of peripheral CD4+ cells following transient depletion

Blood was collected from anti-CD4-treated recipient mice via the saphenous vein at days 7, 14, 21, 28 and 42 posttransplantation and lymphocytes were isolated over a Ficoll–Paque gradient (Amersham Biosciences, Uppsalla, Sweden). CD4+ cells were identified using FITC-labeled anti-CD4 and flow cytometry. Staining with FITC-conjugated rabbit anti-rat IgG (Biosource International, Camarillo, CA) was employed to verify that *in vivo* mAb therapy depleted CD4+ cells, as opposed to masking the CD4 epitope.

Statistical analyses

Allograft survival curves were analyzed using a log-rank test. Significance of ELISPOT and alloantibody results was determined by an unpaired *t*-test with Welch's correction. CD4+ T cell return kinetics were compared using two-way analysis of variance with Bonferroni posttests. All data were analyzed using GraphPad Prism v. 4.0 (GraphPad Software, Inc. San Diego, CA) and p values $\leq$0.05 were considered statistically different.

Results

Allograft survival is not prolonged in C1q-/- recipients

In order to determine if C1q deficiency affected the tempo of rejection, C1q-/- mice were used as recipients of BALB/c cardiac allografts and the time of rejection was compared to WT recipients. Figure 1 illustrates that C1q deficiency was not protective in the context of cardiac allograft rejection, as C1q-/- recipients acutely rejected their grafts. Indeed, C1q-/- recipients rejected their allografts at a significantly faster tempo (mean survival time = 7.5 days $\pm$ 0.5; $p < 0.01$) than did WT recipients (mean survival time = 9 days $\pm$ 1). These results indicate that deficiency in

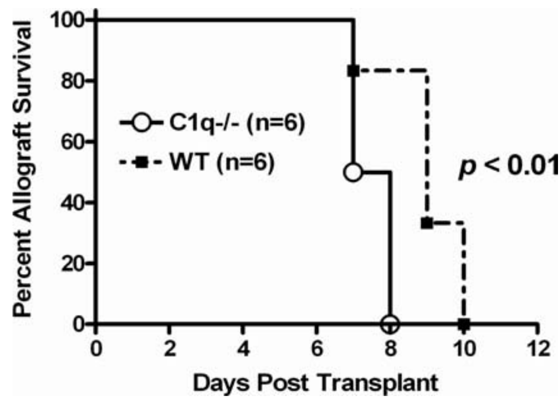
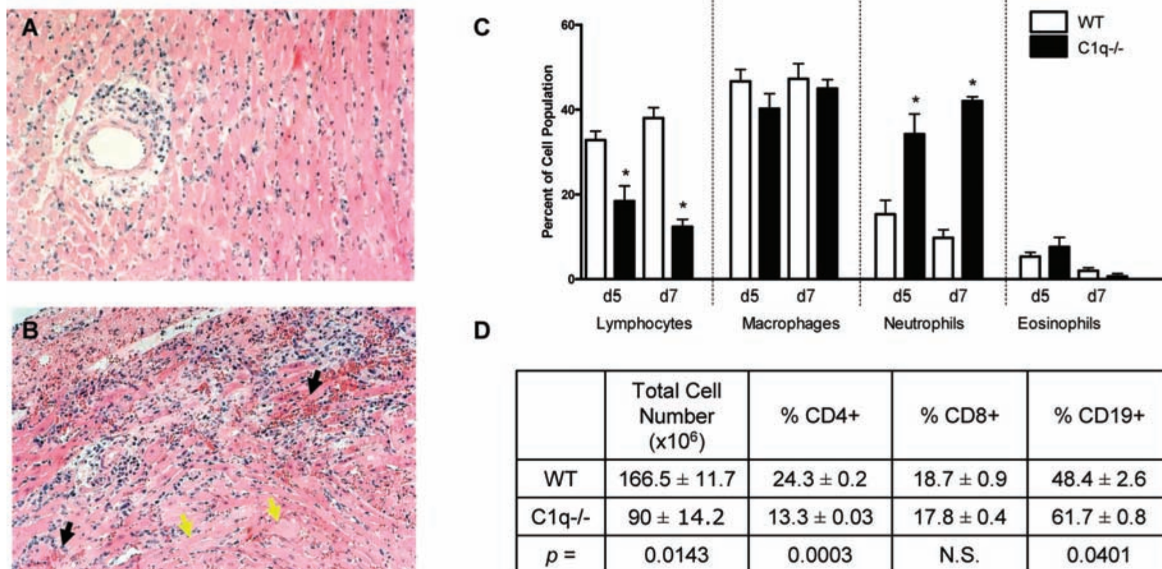


Figure 1: C1q deficiency does not delay allograft rejection. WT and C1q-/- mice were transplanted with WT BALB/c cardiac allografts. The number of transplants per experimental group is given in parentheses. Transplant function was evaluated by daily palpation, and the day of rejection was recorded as the day the transplant ceased functioning.

the classical pathway of C activation may, in fact, be detrimental to cardiac allograft survival and suggest a protective role for C1q in the rejection process.

Increased severity of rejection in C1q-/- allograft recipients

To assess the severity of the rejection response in WT and C1q-/- recipients, allografts were recovered on day 7 post-transplantation and evaluated histologically. At this time, early signs of rejection were observed in allografts from WT recipients, including a diffuse mononuclear cell infiltrate and mild arterial inflammation (Figure 2A). A more intense cellular infiltrate was observed in the allografts of C1q-/- mice (Figure 2B), which was accompanied by hemorrhage (black arrows) and extensive myocyte necrosis (yellow arrows). Wright's stained differential counts of GIC isolated from C1q-/- and WT recipients revealed differences in infiltrate composition. On day 5, GIC in the grafts of C1q-/- recipients were primarily composed of neutrophils and macrophages with < 20% of the infiltrate lymphocytes (Figure 2C). In contrast, GIC isolated from WT recipients contained mostly lymphocytes and macrophages. The increased percentage of neutrophils persisted to day 7 in grafts of C1q-/- recipients. Flow cytometry analysis of splenocytes revealed a significant 23% increase in the percentage of CD19+ B cells and a 45% decrease in CD4+ T cells in C1q-/- recipients as well as an overall decrease in the number of total splenocytes compared to WT recipients (Figure 2D). Together, these data suggest that C1q deficiency alters the immune response to the transplant.



	Total Cell Number ($x10^6$)	% CD4+	% CD8+	% CD19+
WT	166.5 ± 11.7	24.3 ± 0.2	18.7 ± 0.9	48.4 ± 2.6
C1q-/-	90 ± 14.2	13.3 ± 0.03	17.8 ± 0.4	61.7 ± 0.8
p =	0.0143	0.0003	N.S.	0.0401

Figure 2: Exacerbated pathology of rejection in C1q-/- mice. Cardiac allografts were harvested on day 7 posttransplantation from WT and C1q-/- recipients and prepared for histologic analysis with H & E staining. In WT recipients (panel A), allografts were functioning on day 7 but exhibited early signs of rejection including a diffuse infiltrate and arterial inflammation. Allografts in C1q-/- recipients (panel B) were not functioning on day 7. Note the intense infiltrate, areas of hemorrhage (black arrows) and myocyte necrosis (yellow arrows). Magnification = 200X. Wright's stained differential counts of GIC revealed increased numbers of neutrophils in allografts of C1q-/- recipients (panel C, * indicates $p \leq 0.05$). Flow cytometric analyses of splenocytes for CD4+ and CD8+ T cells and CD19+ B cells were compared between groups of three WT and three C1q-/- transplant recipients (panel D). Total splenocyte yields are also depicted. Significant p values are indicated.

T-cell responses are not enhanced in C1q-/- allograft recipients

To determine if exacerbated allograft rejection in C1q-/- mice was reflected by an enhanced cellular immune response, we employed short-term ELISPOT assays to quantify the number of *in vivo* primed, donor-reactive Th1 (IFNγ) and Th2 (IL-4) in the spleens of allograft recipients. On the day of rejection (Figure 3A), C1q-/- recipients mounted reduced (though not significantly; $p = 0.056$) Th1 responses when compared to WT mice. Th2 responses were negligible in both groups, in keeping with previous reports that unmodified rejection is characterized by a dominant Th1 response in this model (47,54).

Primed donor-reactive T cells may be detected at peak numbers in the spleen prior to allograft rejection (49). Hence, we quantified Th1 and Th2 responses in the spleens of C1q-/- and WT allograft recipients on day 5 posttransplantation (Figure 3B). C1q-/- recipients mounted a significantly reduced Th1 response relative to their WT counterparts ($p < 0.0001$). The Th2 response in both groups was unremarkable. Hence, accelerated rejection in C1q-/- mice was not reflected by an enhanced cellular immune response and may, in fact be associated with a decreased Th1 response.

Accelerated rejection in C1q-/- mice is reflected by an early alloantibody isotype switch and IgG deposition in the graft

Hemorrhage observed in transplants from C1q-/- recipients suggested that an exacerbated alloantibody response may occur in these recipients (55). Hence, we analyzed recipient sera for donor-reactive IgG alloantibodies 5 days posttransplantation and at the time of transplant rejection. Similarly, low levels of donor-reactive IgM were observed in both C1q-/- and WT recipients at both time points (data not shown). However, at 5 days posttransplantation, donor-reactive IgG antibody levels were significantly higher in the sera of C1q-/- recipients when compared to their WT counterparts (Figure 4A). At the time of rejection, donor-reactive IgG was detectable in WT recipients and sera levels approached those seen in C1q-/- mice (data not shown). Thus, early production of donor-reactive IgG correlated with the early onset of rejection in C1q-/- mice.

We next determined if the increased early production of IgG correlated with IgG deposition within the graft. Immunohistochemical analysis of allograft tissue isolated from C1q-/- recipients at day 5 posttransplant revealed intense staining of IgG associated with arteries, with punctate staining in the pericardium and around cardiac myocytes (Figure 4B). In contrast, IgG staining in hearts isolated from WT recipients at day 5 showed very little IgG deposition in the arteries and no staining of myocytes. Deposition of IgG in allografts isolated from C1q-/- recipients persisted until at least day 7. At this time point, a similar pat-

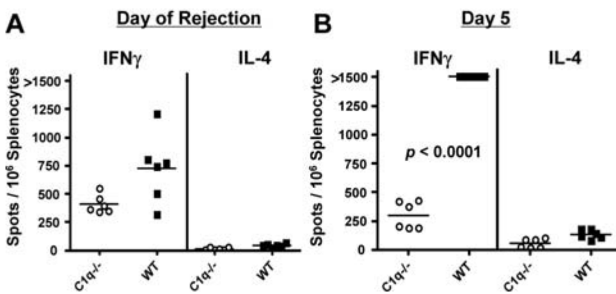


Figure 3: Donor-reactive T-cell responses are not enhanced in C1q-/- allograft recipients. Spleens of transplant recipients were processed into single cell suspensions and stimulated with irradiated WT BALB/c splenocytes in overnight ELISPOT cultures to quantify primed donor-reactive IFNγ (Th1) and IL-4 (Th2) producing cells. Data are presented as the number of spots/10^6 splenocytes and the responses of individual animals are plotted. The horizontal bars represent the mean values for each experimental group. The upper limit of detection for this assay is 1500 spots. Spleens were recovered at the time of transplant rejection (panel A) or at 5 days posttransplantation (panel B).

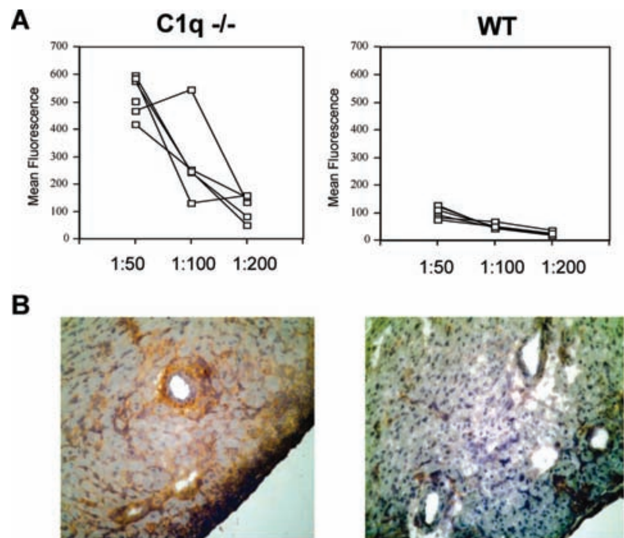


Figure 4: Accelerated production of donor-reactive IgG in C1q-/- allograft recipients. Panel A: Sera were obtained from WT (n = 6) or C1q-/- (n = 6) allograft recipients on day 5 posttransplantation, diluted 1:50, 1:100 and 1:200, and assessed for donor-reactive IgG binding to P815 (H-2^d) cells by flow cytometry as described under 'Materials and Methods'. Data are reported as the mean fluorescence intensity of each dilution of sera for six individual mice per experimental group. Significance for each dilution: 1:50, $p < 0.0001$; 1:100, $p = 0.0304$; 1:200, $p = 0.0128$. Panel B: Fixed tissue sections from heart allografts isolated at day 5 from C1q-/- (left panel) or WT (right panel) recipients were incubated with goat anti-mouse IgG HRP-labeled antibody followed by development with AEC to visualize mouse IgG deposition. Results are representative of 5 grafts from C1q-/- recipients and 3 grafts from WT recipients. 200 × magnification.

tern of IgG deposition was also observed in grafts isolated from WT recipients (data not shown). These results suggested that early deposition of IgG within the transplant may contribute to the accelerated pathology observed in allografts isolated from C1q-/- recipients.

Differential deposition of C3d versus C4d in grafts of C1q-/- and WT recipients

We next investigated the deposition of C3d and C4d within the grafts, which along with antibody deposition is considered a clinical marker of Ab-mediated rejection in transplant (21–26). Immunohistochemical staining revealed intense C4d deposition in the grafts of WT recipients (Figure 5). In contrast, C4d staining in grafts isolated from C1q-/- recipients was negligible. However, there was a significant deposition of C3d on capillaries throughout these grafts. Since deposition of C4 split products results from activation of the classical and/or MBL pathways, deposition of C3 in the allografts suggests a possible role for compensatory alternative pathway activation in C1q-/- recipients (43,48).

C1q requirements for the effectiveness of inductive anti-CD4 and anti-CD40L mAb therapies

It is well established that inductive mAb therapy with either anti-CD4 (GK1.5; that is 49–51) or anti-CD40L (MR1; that is 51,53) mAb prolongs allograft survival. However, the mechanisms by which these therapies prevent rejection are not completely defined. GK1.5 is a C-fixing IgG2b mAb that depletes $CD4^{+}$ T cells *in vivo* (56). We hypothesized that initiation of the classical C pathway through C1q

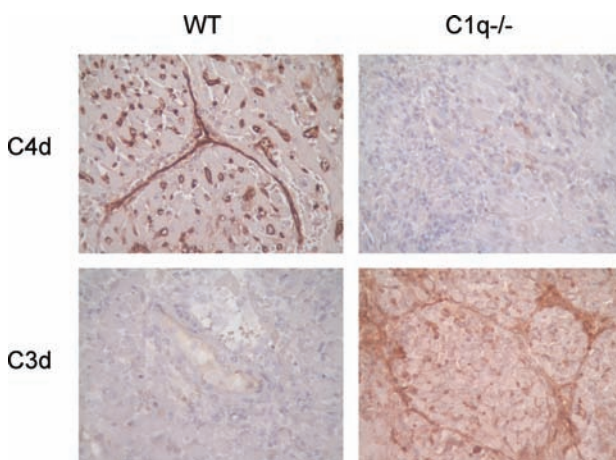


Figure 5: C3d, but not C4d, is deposited in grafts of C1q-/- recipients. Fixed tissue sections from heart allografts isolated at the day of rejection from WT (left panel) or C1q-/- (right panel) recipients were incubated with anti-C4d or anti-C3d followed by the development with DAB to visualize mouse C split product deposition. Results are representative of staining from 4/5 grafts from C1q-/- recipients and 3/3 grafts from WT recipients. 400X magnification.

binding to the GK1.5-CD4 cell complex would mediate the beneficial actions of this mAb in transplantation. Some evidence suggests that the anti-CD40L mAb MR1 may not simply block CD40–CD40L interactions, but may function in part through C-mediated lysis of CD40L-expressing cells (41,42). Hence, we asked whether the absence of C1q would negate the protective activities of anti-CD4 or anti-CD40L mAb therapy. As shown in Figure 6A, WT recipients treated with anti-CD40L mAb maintained their grafts for >60 days. Furthermore, allograft survival was not significantly shortened in C1q-/- mice that were treated with anti-CD40L mAb in that 9 of 12 transplants continued to function normally for >60 days. Hence, C1q and the classical C pathway do not appear to be required for prolonged allograft survival following inductive anti-CD40L therapy in this model.

While anti-CD4 mAb therapy was uniformly protective in WT recipients, it was significantly less effective in C1q-/- mice in that 13 out of 21 animals rejected their allografts before day 60 posttransplant (Figure 6B, $p = 0.01$). However, graft rejection in anti-CD4-treated C1q-/- mice was delayed with a mean rejection time of 37 days. If CD4+ cells were not initially depleted in C1q-/- mice, the prediction would be that anti-CD4 therapy would be ineffective and that rejection would occur at an accelerated tempo. Hence, we determined the efficacy of CD4+ cell depletion in C1q-/- and WT allograft recipients (Figure 6C). Allograft recipients were serially bled at weekly intervals following initial anti-CD4 treatment and peripheral blood CD4+ cell levels were assessed by flow cytometry. In WT recipients, CD4+ cells began to repopulate the periphery between 3 and 4 weeks following initial depletion, consistent with previous studies (50,52). Interestingly, CD4+ cells were effectively depleted in C1q-/- recipients and repopulated the periphery with kinetics similar to those observed in WT mice. Hence, the decreased efficacy of anti-CD4 therapy in C1q-/- allograft recipients was not associated with ineffective depletion of CD4+ cells.

Discussion

The mechanisms by which C1q mediates its protective effects in the context of transplantation are not understood and merit further investigation. Previous reports indicate that C1q and natural antibodies play a role in ischemia/reperfusion (I/R) injury (34,35,37,57). Therefore, one could anticipate that loss of C1q might reduce inflammation following I/R injury to the graft. However, other data suggest that elimination of C1q does not ameliorate inflammation (58). Still more studies implicate the lectin and/or the alternative pathways, rather than the classical pathway, in I/R injury (32,38,39). Further, it has been suggested that the early, enzymatic responses in the C activation pathway may regulate immune responses (1). Finally, C-regulatory proteins have been implicated in modulating adaptive immune responses (59). Hence, both

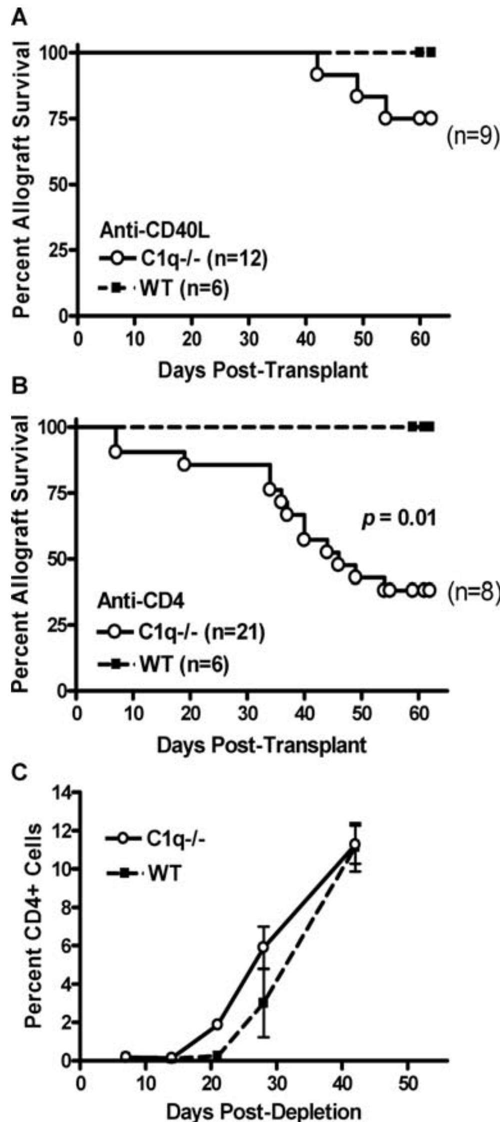


Figure 6: Anti-CD4, but not anti-CD40L mAb therapy is compromised in C1q-/- allograft recipients. WT and C1q-/- mice were transplanted with BALB/c allografts and given inductive anti-CD40L (panel A) or anti-CD4 (panel B) mAb. The total number of allograft recipients in each experimental group is given in parentheses. All allografts in the WT groups were functioning at the termination of the experiment on day 60 posttransplantation. The numbers of grafts in C1q-/- recipients that were functioning on day 60 are given in parentheses. Panel C depicts peripheral CD4+ cell depletion and kinetic repopulation in WT (n = 3) and C1q-/- (n = 3) allograft recipients that received anti-CD4 mAb therapy. Mice were serially bled at the indicated intervals and lymphocytes were isolated and assessed for CD4+ cell content by flow cytometry.

pro- and anti-inflammatory activities of the C system have been described and the complex interactions of these activities within the transplant setting have yet to be determined.

The classical C pathway may in fact be required to prevent inflammation. For example, C1q deficiency promotes the development of SLE in MRL mice (12,13), which has been associated with the inability of macrophages to bind to and clear apoptotic bodies (14,15). Phagocytosis of apoptotic bodies by DC renders them immunosuppressive (60) and feeding DC with donor-derived apoptotic bodies results in the deletion of donor-reactive T cells, suppression of the anti-donor immune response and prolongation of allograft survival (61). Hence, it is possible that the increased pathology of rejection observed in C1q-/- recipients is linked to an inability of recipient macrophages and/or DC to phagocytize apoptotic bodies within the graft, resulting in increased inflammation. To test this possibility, we compared TUNEL staining for apoptotic cells in cardiac allografts obtained from C1q-/- versus WT recipients at the time of rejection (data not shown). No differences were observed between the groups, indicating that differential clearance of apoptotic bodies at the time of rejection was not associated with enhanced pathology. However, this does not rule out a possible role for this process in the early phase of the rejection response.

The donor-reactive Th1 response was significantly reduced in C1q-/- allograft recipients relative to their WT counterparts (Figure 3). It is unlikely that this result is due to differences in splenocyte populations observed in C1q-/- versus WT recipients (Figure 2D), since CD8+ T cells are not reduced in C1q-/- mice and this lymphocyte population is responsible for the majority of IFNγ production in allograft rejection (47). Our data are consistent with a study by Cutler et al. (62), who reported that C1q-/- mice were deficient in IFNγ production in response to T–cell-dependent antigens. These authors propose that a direct interaction between C1q and APC may result in IL-12 release and subsequent Th1 responses. Indeed, macrophages and DC express C1qR and the binding of C1q enhances phagocytic activity (3,63–65). In addition, C1q has been shown to enhance the uptake and MHC class I presentation of antigen associated with immune complexes to $CD8^{+}$ T cells (66). Given these considerations, the muted Th1 responses in C1q-/- allograft recipients may reflect the absence of C1q stimulation of APC function, rather than a direct effect on the T cell.

Accelerated allograft rejection in C1q-/- recipients was associated with an accelerated isotype switch of donor-reactive alloantibodies and deposition of IgG within the graft (Figure 4). Indeed, early onset of hemorrhage observed in the grafts of C1q-/- but not WT recipients (Figure 2) is in keeping with Ab-mediated rejection (55). The underlying mechanisms responsible for this accelerated switch of donor-reactive antibodies to IgG are not readily apparent. However, antibody-mediated depletion of C1q in a mouse model of myasthenia gravis has also been shown to result in increased antibody production and IgG deposition in kidney (67), suggesting an important role for C1q in the regulation of the antibody response.

It is also possible that loss of the classical pathway of C activation in C1q-/- recipients results in a compensatory response by the MBL or the alternative pathways. Indeed, immunohistochemical staining of allografts isolated from C1q-/- recipients revealed that C3d, but not C4d was deposited in the transplants (Figure 5). These results are consistent with published studies documenting that antibodies in C1q-/- serum were unable to fix C and potentiate C4d deposition in cardiac allografts (48). Further, in the absence of C1q, the alternative pathway can activate C3 (43).

We cannot rule out the possibility that our results are due not only to the lack of C1q in the system, but by changes in the levels of other C proteins as well. C1q-/- mice have decreased levels of C4, as well as MBL-A (43). Indeed, C4-deficient mice can deposit C3d on kidney grafts, most likely through the alternative pathway (68). However, a decrease in C4 does not account for the early switch to IgG and increased IgG binding in the graft (Figure 4). In fact, mice deficient in C4 display impaired IgG responses to allogeneic skin grafts (69). In summary, our observations suggest two important effects of loss of C1q: firstly, C1q deficiency results in an earlier switch to and greater production of IgG and secondly, decreased C4 in C1q-/- mice results in compensatory deposition of C3 in the graft. In addition, the high levels of circulating IgG as well as the IgG deposited in the grafts in C1q-/- recipients may enhance C3 activation by the alternative pathway (43,70), leading to the exacerbated rejection response in this system.

This study also assessed the contribution of C1q and the classical C pathway to the protective activities of the widely used experimental mAb, anti-CD40L (MR1) and anti-CD4 (GK1.5). *In vivo* treatment of cardiac allograft recipients with either of these mAb results in prolonged graft survival (49-51, 53). While previous reports (41,42) indicate that the protective activity of anti-CD40L mAb is at least in part dependent upon C, we found that this activity was not significantly dependent upon C1q and the classical C pathway (Figure 6A). *In vitro*, cross-linking CD40L has been reported to alter human T-cell function resulting in the production of IL-4 and IL-10 (71) as well as inducing apoptosis of activated T cells (72). Hence, the suppressive mechanisms of action of anti-CD40L mAb are likely multi-factorial.

In contrast to anti-CD40L, anti-CD4 mAb therapy was significantly less effective in C1q-/- recipients (Figure 6B), in keeping with the notion that the suppressive activity of anti-CD4 mAb is related to C-mediated depletion of CD4+ T cells. However, transient depletion of CD4+ cells from the circulation was not affected by C1q deficiency and the kinetic return of CD4+ cells to the periphery was similar in WT and C1q-/- recipients (Figure 6C). This observation was unanticipated since the anti-CD4 mAb GK1.5 is a C fixing rat IgG2b and facilitates C-mediated lysis of CD4+ cells *in vitro* (56). However, *in vivo* depletion of CD4+ cells by GK1.5 has also been reported in C5-deficient mice (56). Thus, while the immunosuppressive effects of the anti-CD4 mAb GK1.5 are associated with transient depletion of CD4+ T cells, depletion occurs independent of C1q and the classical C pathway. What is not clear is how C1q may influence the hyporesponsive state of CD4+ cells once they repopulate the periphery following the initial peritransplant depletion. In WT mice, graft-reactive cells repopulate the periphery after initial depletion yet are maintained in a quiescent precursor state (50). In C1q-/- mice, repopulation of the periphery by CD4+ cells is associated with allograft rejection in a significant number of allograft recipients (Figure 6B), implying that C1q contributes to induction of the hyporesponsive state of returning CD4+ cells. This failure to induce graft acceptance may be associated with decreased donor-reactive Th1 priming in C1q-/- allograft recipients (Figure 3), as IFNγ has been reported to be required for long-term graft acceptance following costimulatory blockade (51,73).

In summary, this study uncovers unanticipated protective activities for C1q in the context of transplantation. While the deposition of C split products within rejecting allografts may indeed have diagnostic implications (1,19,74), the anti-inflammatory contributions of the early C components may prove to be critical for allograft survival.

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