Deficiency of C4 from Donor or Recipient Mouse Fails to Prevent Renal Allograft Rejection

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Complement effector products generated in the transplanted kidney are known to mediate transplant rejection, but which of the three main activation pathways of complement trigger this response is unclear. Here we assessed the role of the classical and lectin pathways by studying the common component C4 in mouse kidney transplant rejection. We transplanted wild-type or C4-null H-2^b donor kidneys into H-2^k or H-2^d recipients, or vice-versa, to assess the roles of donor kidney and recipient expression of complement. Intra-graft C4 gene expression rose substantially during rejection. However, we found no significant association between graft acceptance and the presence of C4 in either the donor kidney or recipient mouse. At the time of rejection, we found no significant differences in alloantibody response in the different groups. Tubular deposition of C3 to C9 occurred regardless of the absence or presence of C4 in either the donor or recipient mouse, indicating that C4 was dispensable for complement activation at this site. These data suggest that complement activation and renal allograft rejection are independent of the classical and lectin pathways in these models, implying that in the absence of these pathways the alternative pathway is the main trigger for complement-mediated rejection. (Am J Pathol 2006; 168:1241–1248; DOI: 10.2353/ajpath.2006.050360)

Complement activation is a well recognized cause of tissue injury in inflammatory conditions including organ transplant rejection.¹ The main complement effector products are 1) the soluble fragments including C3a and C5a, which mediate inflammation; 2) the membrane at-

tack complex C5b-9, which causes membrane injury leading to cell activation and cell death; and 3) the large, surface-bound fragments of C3 that mediate immune cell adhesion.² Formation of these products requires the cleavage of C3, which can be initiated by the classical, alternative, and lectin pathways.

The fourth component is an essential intermediary for the classical and lectin pathways of complement activation. The classical pathway is triggered by antibody and C1q binding to target structures, resulting in the cleavage of C4. In addition, C1q can bind directly to activating surfaces, such as apoptotic blebs, nuclear material, and C-reactive protein, bypassing the need for antibody to initiate the cleavage of C4.^{3,4} In contrast, the lectin pathway uses mannose-binding lectin and associated serine proteases to initiate the cleavage of C4.⁵ Once cleaved by the classical or lectin pathways, C4 attaches to activator surface, forms a complex with C2 that activates C3, and subsequently leads to membrane insertion of complement. In addition, bound C4 acts as a ligand for complement receptor CD35 expressed on leukocytes.⁶ The subsequent degradation product C4d has no known biological function but serves as a marker of humoral allo-rejection.^{7,8} The soluble fragment C4a has only weak proinflammatory properties. Because each bound molecule of C4 can result in up to 10 molecules of attached C3, C4 constitutes an important amplification and regulatory step in the complement cascade.

Although most circulating complement is produced by hepatocytes, extrahepatic synthesis occurs at several tissue locations. Important local sources of C3 and C4 include resident tissue cells (eg, epithelial cells, endothelial cells, and fibroblasts) and migratory leukocytes (eg, neutrophils and macrophage/monocytes).^{9,10} Recent work in a renal allograft model has shown that intrarenal production of C3 was essential for graft rejection.¹¹ Transplants from C3-deficient mouse donors had less inflammation and generated a weaker recipient immune

Supported by grants from the Medical Research Council, UK, and Wellcome Trust, UK.

Accepted for publication December 29, 2005.

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response than transplants from wild-type donors. Because C4 is needed for two of the three major pathways leading to C3 activation, we aimed to define the contribution of C4 in the mechanism of allograft rejection. This is important because selective inhibition might allow limited therapeutic blockade with fewer unwanted effects.

We tested the hypothesis that C4 was a determinant of acute allograft rejection. Our prediction was that deficient local or systemic production of C4 would lead to increased graft acceptance. For this purpose, we used a known complement-sensitive model of renal allograft rejection (C57BL/6 donor to B10.Br recipient) and in addition investigated two other donor-recipient strain combinations.

Materials and Methods

Mice

Male C57BL/6 (B6; H-2^b), B10.Br (H-2^k), BLAB/c (H-2^d), and C3H/HeN (H-2^k) mice were obtained from Harlan Olac (Bicester, UK). The C4^{-/-} mice were derived by homologous recombination in embryonic stem cells¹² and backcrossed onto the C57BL/6 parental strain for 11 generations. C4 mRNA and protein is undetectable in these mice by real-time polymerase chain reaction (PCR) or Western blotting. All procedures were conducted in accordance with the Home Office Animal (Scientific Procedures) Act of 1986.

Renal Transplantation

Donor mice, 6 to 8 weeks of age, were anesthetized and the abdomen was opened through a midline incision. The left kidney was excised and preserved in cold saline. Recipient mice were then anesthetized and the right native kidney was excised. Renal transplantation was performed with end-to-side anastomoses of the donor renal vein to the inferior vena cava and the donor aortic cuff to the aorta.¹³ Urinary tract reconstruction was accomplished using ureter-to-bladder anastomosis.¹⁴ No immunosuppressive therapy was administered at any time during the experiment. The left native kidney was removed at 1 week after transplantation. We placed the graft in the right flank because left nephrectomy is technically easier to perform. After that, renal function was determined by measuring blood urea nitrogen at days 8, 10, 12, 14, and 16 and then once a week. The end-point of graft survival was taken as the time to blood urea nitrogen >50 mmol/L or death, depending on which was first.

RNA Isolation and Real-Time Reverse Transcriptase (RT)-PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen Ltd., West Sussex, UK). Five μ g of total RNA was reverse-transcribed into cDNA using the M-MLV reverse transcriptase. Quantification of gene expression was per-

formed using DNA Engine Opticon System (MJ Research, Cambridge, MA). C4 primer was designed using the computer program Oligo 4 (National Biosciences Inc., Plymouth, MN), and the sequence is forward 5'-GGATCCAGCAGTTTCGAAAG-3', reverse 5'-ACTGGACATGGG TCGTGGAA-3'. Melting curves were used to detect primer-dimer conformation and nonspecific amplification. Threshold cycle (Ct) numbers were determined and transformed using the Δ Ct and $\Delta\Delta$ Ct methods as described by the manufacturer, using GAPDH as the internal control.

In Situ Hybridization

Mouse C4 mRNA was performed on frozen sections using the HybriProbe *in situ* hybridization assay kit (Biogenostik, Göttingen, Germany). The mouse C4-specific oligonucleotide was derived from the published sequence (accession number: NM.009780). Briefly, 4- μ m frozen sections were fixed in 4% paraformaldehyde on silanized slides (DAKO, High Wycombe, UK). Sections were pre-hybridized for 4 hours, hybridized for 16 hours with fluorescein isothiocyanate (FITC)-labeled probe for C4 mRNA, and then washed with standard saline citrate. Bound probe was detected by immunochemical staining using an alkaline phosphatase-conjugated F(ab') antibody fragment to FITC (DAKO) followed by the substrate mixture BCIP/NBT (DAKO). This resulted in purple product at the site of hybridization. Slides were then counterstained with hematoxylin for 10 seconds.

Histological Examination of Renal Tissue

Renal tissue was excised and fixed in 4% formalin and embedded in paraffin. For histological analysis, 4- μ m sections were mounted on slides and stained with periodic acid-Schiff (PAS). The sections were reviewed in a blinded manner by two experienced persons for evaluation of rejection.

Immunofluorescence

Four- μ m frozen sections of the renal allografts were stained for C4, C3d, and C9 using an indirect immunofluorescence method. The slides were fixed in acetone and then blocked in normal goat serum. The primary antibodies for C3 and C9 staining were rabbit anti-human C3d (dilution 1:200; Dako Ltd., Cambridgeshire, UK) and rabbit anti-rat C9 (dilution 1:100; generously provided by Dr. B. Paul Morgan, University of Wales). The secondary antibody was a FITC-conjugated polyclonal goat anti-rabbit IgG (dilution 1:200; Jackson ImmunoResearch, West Grove, PA). For C4 staining, rat monoclonal antibody to C4 (dilution 1:50; Abcam Ltd., Cambridge, UK) and FITC-conjugated goat anti-rat IgG (dilution 1:200; ICN Biomedicals Inc, Aurora, OH) were used. C3- and C9-positive staining was quantified using Lucia image analysis software (Jencons-PLS, Forest Row, UK). At a magnification of $\times 400$, for each animal, six fields from two stained kidney sections were photographed. Areas of

positive staining in each image were outlined, highlighted, and values computed.

Flow Cytometry

The presence of circulating donor-specific IgM and IgG antibodies were evaluated in the recipient serum by flow cytometry (FACScan; BD Biosciences, Cowley, UK). Naïve donor-strain mouse splenocytes were isolated and resuspended in 50 μ l of phosphate-buffered saline + 2% bovine serum albumin at a concentration of 1×10^7 /ml. The splenocytes were stained with a phycoerythrin-conjugated antibody specific for CD3 (BD Biosciences), followed by incubation with the sera from recipients. Then the cells were incubated with FITC-conjugated goat antibody specific for mouse IgM or IgG (Sigma, Dorset, UK). Each incubation was performed at 4°C for 20 minutes. The cells were washed, fixed, and analyzed by two-color flow cytometry gated on T cells. The mean fluorescence intensity of each test sample was compared with that obtained from serum of a naïve mouse and the relative mean fluorescence was determined. Specificity of alloantibody binding was assessed by testing the serum against recipient strain splenocytes. Preliminary studies using a titration of serum against donor splenocytes found the concentration of 1:25 had a linear relationship with mean fluorescence intensity, which was used in all subsequent experiments.

Statistical Analysis

Survival analysis between groups was calculated using the log-rank method. Complement C3 and C9 deposition were analyzed using one-way analysis of variance. Antibody production was assessed by the Mann-Whitney test. All results were generated using GraphPad Prism software (GraphPad, San Diego, CA). Statistical significance was considered by a *P* value <0.05.

Results

Intragraft Expression of C4 Is Increased in Renal Allograft Rejection

Wild-type B6 (H-2^b) donor kidneys transplanted into B10.Br (H-2^k) recipients were rejected acutely, with graft loss occurring by day 9 after transplantation in all cases. Real-time PCR analysis of day 8 renal transplants is shown in Figure 1. The results indicate that the amount of C4 transcript in rejecting grafts was increased by 2 to 3 logs. Localization studies with C4 mRNA probe are illustrated in Figure 2, A and B. The renal tubule is seen to be the main site of C4 expression in normal C57BL/6 kidney. Immunofluorescent staining for tissue C4 is presented in Figure 2, C and D. It shows evidence of C4 deposition in the peritubular capillary walls of rejecting grafts.

Figure 1. Complement C4 mRNA expression in normal B6 kidneys and rejecting B6 grafts. Real-time RT-PCR of C4 transcripts in rejecting B6 grafts. C4 mRNA levels are expressed as fold increase over normal B6 kidneys. Each analysis was performed on RNA isolated from three individual kidneys, and assays were run in duplicate.

C4-Null Kidney Transplants Are Rejected Acutely

To assess the influence of intragraft complement gene expression on acute rejection, we determined graft survival times with C4^{-/-} B6 donor kidney transplanted into wild-type B10.Br recipients. The results are illustrated in Figure 3. Acute rejection occurred in 80% of C4^{-/-} donor kidneys, compared with 100% for C4^{+/+} (wild-type) donor grafts. This difference is not significant. Graft histology at rejection is shown in Figure 4, A and B. The extent

Figure 2. Localization of C4 expression in wild-type donor kidney. **A and B:** In the wild-type normal B6 kidney, *in situ* hybridization of C4 mRNA showed positive staining in the cytoplasm of tubule cells. There was no staining found in C4^{-/-} kidney tissue. **C:** Immunofluorescence staining for C4 in allografts showed staining predominantly of peritubular capillary walls. **D:** Background C4 staining in normal B6 kidney tissue. Original magnifications, $\times 250$.

Figure 3. Effect of donor C4 status on graft survival: B10.Br recipients of wild-type kidney reject their transplants within 9 days. Eight of ten B10.Br recipients of C4^{-/-} grafts rejected their transplants in 9 days; the other two recipients did not acutely reject but survived until killing at day 72 or day 110. *P* = 0.42 comparing C4^{-/-} and wild-type grafts.

of tubulointerstitial infiltration in rejected C4^{-/-} donor kidneys was not obviously different from that in rejected C4^{+/+} kidneys. C4 staining was still clearly detected in the peritubular capillary walls of C4^{-/-} grafts (Figure 4C), indicating that recipient protein was the source of the capillary C4 staining. Residual C4 mRNA was detected in rejecting C4^{-/-} grafts (Figure 5); this could have been the result of expression by infiltrating leukocytes¹⁵ or

Figure 4. Histology and C4 staining in the rejecting B6 grafts. **A:** Typical rejecting kidney allograft (B6 to B10.Br) at day 8 showing heavy interstitial infiltration, venulitis, and tubulitis (→) (PAS stain). **B:** Typical rejecting C4^{-/-} kidney graft at day 8 showing a similar pattern to B6 wild-type graft (PAS stain). **C:** Diffuse C4 deposition was detected in the peritubular capillary walls of C4^{-/-} grafts, which was not present in normal nontransplanted C4^{-/-} kidney (**D**). a, Arteriole; v, vein; t, tubule. Original magnifications, ×250.

Figure 5. Real-time RT-PCR of C4 transcripts in rejecting grafts. C4 mRNA levels are expressed as fold increase over normal B6 kidneys. Three samples were used in each analysis, and assays were run in duplicate.

contaminating blood cells. As for the surviving C4^{-/-} grafts, except for infiltration around the major arteries the histology was no different from normal tissue (data not shown). In addition, the renal function of long-time surviving recipients, as measured by blood urea nitrogen, was normal.

C4-Null Recipients Reject Wild-Type Grafts

We next examined the effect of circulating C4, by transplanting C4^{-/-} B6 recipients with donor kidneys from B10.Br mice. Graft survival data are presented in Figure 6. With C4^{-/-} recipients the rate of acute rejection was 90%, compared to 80% for wild-type recipients. Thus, the hypothesis that deficient recipient C4 would prevent graft rejection was unsupported. Histological analysis at rejec-

Figure 6. Effect of recipient C4 on graft survival. In B10.Br to B6 group, two recipients were sacrificed at days 56 and 82; the others rejected their grafts at day 8 or day 9. C4^{-/-} recipients rejected 9 of 10 B10.Br grafts before day 9. Another recipient was killed at day 34.

Figure 7. Histology and C4 staining in the rejecting B10.Br grafts. **A** and **B**: B10.Br allografts in wild-type and $C4^{-/-}$ B6 recipients at day 8 showing similar pattern of interstitial infiltration, venulitis, and tubulitis ($\rightarrow$) (PAS stain). **C**: Immunofluorescence staining for C4 in B10.Br to $C4^{-/-}$ allografts showed background staining. Original magnifications, $\times 250$.

tion showed no difference in the extent of tubular damage and leukocyte infiltration (Figure 7, A and B). There was no deposition of C4 in the peritubular capillaries (Figure 7C), which again proved that systemic C4 was the source of staining in the wild-type recipient experiments. The surviving $C4^{+/+}$ grafts displayed normal features, except that infiltrating cells were present around some of the larger arteries.

Different Donor-Recipient Strain Combinations Show No Effect of C4 from Donor or Recipient

The B6 to B10.Br transplant model described above exhibited marked cell-mediated rejection, supporting the suggestion that B10.Br mice have a predominant Th1 response. We next transplanted $C4^{+/+}$ or $C4^{-/-}$ B6 donor kidneys into BALB/c ($H-2^d$) mice. These are reported to have a Th2 phenotype that favors an antibody-mediated response.^{16,17} The graft survival data are shown in Figure 8. There was no clear difference in graft survival between the two sets of donor kidneys. Histological data from rejecting grafts is illustrated in Figure 9, A and B. This indicates features of cell-mediated rejection in both groups of transplants.

We also examined strain combinations involving C3H/HeN ($H-2^k$) mice. C3H reportedly give weaker transplant rejection, which might allow detection of any subtle effects of C4.¹⁸ The outcome of these transplant experiments is summarized in Table 1. When either the transplant donor or recipient was $C4^{-/-}$, acute rejection occurred in five of six recipients. This was not signifi-

Figure 8. Kidney transplant rejection in BALB/c recipients. Seven of eight BALB/c recipients of $C4^{-/-}$ grafts rejected their transplants within 9 days. The other one was killed at day 43. In B6 to BALB/c group, acute rejection occurred in every case.

cantly different compared to the respective wild-type donor or recipient controls.

Alloantibody Response Is Unaffected by C4 at Rejection

Previous studies have shown that complement-deficient mice have impaired antibody responses.¹⁹ To investigate if this is the case in our model, the donor-specific IgG and IgM alloantibody responses were determined by flow cytometry. Because the majority of recipients failed to survive more than 9 days after transplantation, serum

Figure 9. Histology of rejecting B6 graft. **A**: Rejecting kidney allograft (B6 into BALB/c) at day 8 typically showing a heavy interstitial infiltration, venulitis, and tubulitis ($\rightarrow$) (PAS stain). **B**: Typical rejecting $C4^{-/-}$ kidney graft at day 8 showing a similar pattern to B6 wild-type graft (PAS stain). Original magnifications, $\times 250$.

Table 1. Survival of Allografts in C57BL/6 and C3H/HeN Strain Combination

Groups	n	Survival (days)
B6 $\rightarrow$ C3H/HeN	6	$8 \times 4, 9 \times 2^*$
$C4^{-/-} \rightarrow$ C3H/HeN	6	$8 \times 4, 9 \times 1, 41^\dagger$
C3H/HeN $\rightarrow$ B6	6	$8 \times 3, 9 \times 2, 30^{*\dagger}$
C3H/HeN $\rightarrow$ $C4^{-/-}$	6	$8 \times 2, 9 \times 2, 10 \times 1, 36^\ddagger$

* $P = 0.44$ versus $C4^{-/-}$ grafts.

$^\dagger P = 0.71$ versus $C4^{-/-}$ recipients.

‡ Sacrifice at the end of experiment.

Figure 10. Donor-specific alloantibody response. IgG and IgM alloantibodies were measured by flow cytometry in serum from recipients 8 days after transplantation. **A–C:** Detection of alloantibodies in recipients of different strain combination, $P > 0.05$ when comparing IgG or IgM in every pair of groups. **D:** Individual IgG responses in C4^{+/+} or C4^{-/-} B6 recipients of B10.Br grafts.

obtained at day 8 after transplantation was used for measurement. As shown in Figure 10, we detected alloantibodies in every group tested. BALB/c recipients had the strongest antibody response, as expected. However, in both B10.Br and BALB/c recipients, the IgG and IgM alloantibody response was independent of donor C4.

This indicates that the alloantibody response is not impaired by deficient local expression of C4. In C4^{+/+} and C4^{-/-} B6 recipients of B10.Br donors, IgM alloantibody production was not significantly different. For IgG production, although two of the C4^{+/+} B6 recipients displayed a relatively strong alloantibody response (Figure 10D), there was no significant difference between the two groups at this time point ($P = 0.17$).

Complement Activation Is Independent of Donor and Recipient C4

To assess local complement activation, we studied the distribution of deposited C3d and C9 in the transplanted kidneys. The results are presented in Figure 11 (data only shown in the B6 and B10.Br strain combination). The mean stained area/section of C3 staining are 1.13 ± 0.33 mm² (normal B6), 4.07 ± 0.89 mm² (B6 to B10.Br), 4.22 ± 0.97 mm² (C4^{-/-} to B10.Br), and 4.21 ± 0.91 mm² (B10.Br to C4^{-/-}). For C9 staining, the values are 0.94 ± 0.34 mm², 3.48 ± 0.84 mm², 3.15 ± 0.74 mm², and 3.35 ± 0.79 mm², respectively. Regardless of whether the donor or recipient was C4^{+/+} or C4^{-/-}, microscopic evaluation of the C3 or C9 deposit showed no obvious differences for all of the groups of transplanted mice ($P > 0.05$), which have larger stained areas ($P < 0.01$) and increased staining intensity compared with normal tissue controls. These results suggest that complement activation through C3 to C9 does not require the presence of either local or systemic C4 in this model.

Figure 11. Complement detection in rejecting renal allografts. **A:** Strong, diffuse C3d deposition was detected in the distribution of the tubular basement membrane and peritubular capillaries of rejecting grafts in all of the groups. **B:** Staining for C9 in rejecting grafts showed a similar pattern of staining as C3d. Original magnifications, $\times 250$.

Discussion

In a total of 48 transplants in which the donor kidney was from a C4-positive or -negative mouse, our results show that the graft survival rate for the C4-negative transplants was not increased. Furthermore, in another 32 transplant procedures, when the recipient was either C4-positive or -negative, the negative recipients showed no graft survival advantage. Thus C4, whether expressed in the donor kidney or recipient, was dispensable in this model of acute rejection.

All graft tissues contained a deposit of C3d and C9 in the region of the tubular basement membrane regardless of strain combination, indicating that complement activation and subsequent membrane insertion did not require the presence of C4. Because the classical and lectin pathways depend on C4 for the formation enzyme complexes capable of cleaving C3, our data suggest no major role for the classical and lectin pathways leading to C3 cleavage and complement insertion. Instead, our data imply that the alternative pathway was responsible for C3 cleavage, in the absence of the classical and lectin pathways. Once cleaved, C3 leads to the formation of C3a, C3b, C5a, and C5b-9. Proinflammatory and immunoregulatory functions downstream of C3 cleavage have recently been shown to mediate renal transplant rejection in a B6 to B10.Br allograft model.¹¹ The data presented here infer a role for the alternative pathway in driving these functions of complement.

The effector function of C4 in acute antibody-mediated rejection is well recognized.²⁰ In the presence of bound antibody and C1, activation of C4 leads to the cleavage of C3 and consequently to the formation of effector products that mediate endothelial injury at the site of Ig and C1 binding. Circulating C4 might be expected to exert greater influence on vascular rejection than locally produced C4, because of the abundance of C4 in the circulation and in view of the contact of blood with endothelium. However, mice with deficient circulating C4 did not exhibit lower rejection rates compared to wild-type mice.

In addition to its effector function, complement can also regulate the B cell response.²¹ For example, Marsh and colleagues²⁰ grafted C3- or C4-deficient mice with allogeneic donor skin and found that complement deficiency impaired the conversion of IgM to IgG alloantibody responses. In the present study, we were unable to detect a difference in the alloantibody response at day 8 after transplantation, with the exception of two recipient wild-type mice. However, primary B-cell responses generally require 14 days to achieve peak serum antibody level²² and the study by Marsh and colleagues²⁰ only detected a difference between complement-deficient and wild-type animals at the peak of the immune response, which was 28 days after skin grafting. Thus, our antibody data collected at 8 days after renal transplantation do not contradict the results for the skin-grafted mice²⁰ but suggest that at the time of rejection C4-mediated regulation of the alloantibody response had no impact on our results. Despite the detection of alloantibodies in each strain combination, the dominant tissue pathology was cell-mediated injury. Taken together with

the lack of effect of circulating C4 on graft survival, this suggests that the predominant mechanism of rejection was cell mediated and that this was independent of C4.

Spontaneous acceptance of grafted tissue in some mouse strain combinations has been recognized since the 1970s.^{23,24} The rate of spontaneous graft acceptance in mice is also donor-tissue related, given that kidney allograft acceptance rates exceed those for cardiac and skin allografts.^{18,25} Recent research has shown that mouse kidney allograft survival rates vary from 20 to 80% depending on the donor-recipient strain combination.^{18,26} The mechanism behind such survival remains unproven. Here, we observed it again in all of the three strain combinations tested in our study. However, the spontaneous acceptance rate was no different when either the donor or recipient was C4-negative. Therefore, it is unlikely that C4 has any effect on spontaneous acceptance.

The apparent lack of C4 at the tubular basement membrane in the presence of bound C3 suggests that C3 cleavage at this site occurred by a C4-independent pathway. Low production of C4 is unlikely to have been a limiting factor, because there was marked up-regulation of C4 mRNA localized to the tubular epithelium in rejecting grafts, and proximal tubule cells are known to efficiently translate and secrete expressed C4.^{27,28} Although several mouse strains are reported to have low generation of C4 as a result of posttranscriptional regulation,²⁹ all our comparisons used a C4-high strain (B6) against C4 knockout mice. Thus, comparison was between high and absent C4 in the donor or recipient arrangements used. Furthermore, even when the recipient was B10.Br, which has been described as a low producer,³⁰ the circulating protein was sufficient to allow C4 deposition on the capillary wall. Therefore, although we cannot fully exclude the possibility of low production causing the lack of tubular C4 protein staining, our immunohistological and survival data strongly suggest that C4 cleavage did not participate in the activation of intrarenal C3. Antibody detection of C4 in the peritubular capillary walls adjacent to negatively stained tubules provided an internal control showing that the marker antibody used was capable of detecting bound C4.

In conclusion the data presented here deny a significant role for C4 leading to complement activation and graft rejection in a mouse renal allograft model. Complement activation at the tubular basement membrane in the absence of either locally or systemically derived C4 suggests an effect independent of the commonly described classical and lectin pathways, which both involve C4. Rather our data suggest that a C4-independent pathway, such as the alternative pathway, is the main driver of complement-mediated rejection in our model.

Acknowledgment

We thank Dr. P. O'Donnell for kindly providing expertise in the histopathology analysis.

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