Deficiency of C5aR Prolongs Renal Allograft Survival
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ABSTRACT
Interaction between C5a, a product of complement activation, and its receptor (C5aR) upregulates antigen-specific T cell responses by modulating the activation of antigen-presenting cells and T cells. Whether this C5a-C5aR interaction contributes to the immune responses that promote renal allograft rejection is unknown. Here, we found that deficiency of C5aR in both graft and recipient reduced allospecific T cell responses and prolonged renal allograft survival. In addition, lack of C5aR impaired the function of donor and recipient antigen-presenting cells and inhibited the response of recipient T cells to allostimulation. Furthermore, deficiency of C5aR in both graft and recipient reduced early inflammation in the grafts, with less cellular infiltration around the vessels and fewer F4/80 positive cells in the peritubular interstitium. These data demonstrate that C5aR is critical for a full adaptive immune response and mediates renal allograft rejection. Engagement of C5aR on dendritic cells and T cells modulates their function, enhancing allospecific T cell responses that lead to allograft rejection. Targeting C5a signaling may have therapeutic potential for T cell–mediated graft rejection.


Recent research has emphasized the importance of the innate immune system in the establishment of adaptive immunity, of which the complement system is a good example. The complement system is a key component of innate immunity. Complement activation, in response to infection or tissue injury, generates a set of effector molecules that have diverse biologic functions, including opsonization of pathogens through the large fragment C3b and its metabolites (iC3b, C3dg), induction of inflammatory responses by the small fragments C3a, C4a, and C5a, and direct killing of pathogens by the membrane attack complex (C5b-9). In addition to their role in innate immunity, several effector molecules including C3a and C5a have been shown to regulate adaptive immunity through activation of their receptors on immune cells.

The small fragments C3a, C4a, and C5a released by complement activation are anaphylatoxic peptides. Of these mediators, C5a has the highest specific biologic activity. It acts as a cell activator with nanomolar affinity, exerting biologic functions through binding of the specific receptor C5aR, a protein G-coupled seven transmembrane domain receptor. Expression of C5aR has been reported in many cell types including both myeloid and non-myeloid cells. It is well known that engagement of the receptor on several myeloid cells (e.g., neutrophils, monocytes/macrophages, and mast cells) leads to the induction of local inflammation through a process of cellular degranulation, increased vascular permeability, and leukocyte recruitment to the site of injury/infection, whereas engagement of the receptor on parenchymal cells (e.g., endothelial cells and epithelial) lead to cell activation and functional modulation.

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Although C5a-induced inflammation plays an important role in the host defense, many studies have shown that C5a can also cause undesired inflammatory responses that contribute to the pathogenesis of several inflammatory diseases such as sepsis, asthma, and ischemia/reperfusion injury. Emerging evidence suggests that C5a also participates in regulating adaptive immune responses. For example, antiviral CD8+ T cell responses were greatly reduced in C5aR antagonist–treated mice, and an enhanced CD8+ T cell immunity phenotype in decay-accelerating factor (DAF, a key complement inhibitor protein) deficient mice was completely rescued by C5aR deficiency. In addition, local production and activation of complement, through its influence on antigen-presenting cell (APC)/T cell interactions modulated by C5a-C5aR engagement, led to enhanced antigen-specific T cell responses and limited antigen-induced T cell apoptosis. Furthermore, it has been shown that C5aR is expressed on DCs and T cells. The expression is upregulated in DCs in response to pathogenic or inflammatory stimuli (e.g., LPS and prostaglandin E2) and in T cells when encountering APCs. These findings suggest that C5aR-ligand interactions can upregulate antigen-specific T cell responses by modulating the activation of APCs and T cells. Therefore, C5a-C5aR interactions may be of significant importance for eliciting specific T cell responses against infection. Furthermore, in certain immunological conditions such as transplant rejection, C5a-C5aR interactions may promote T cell–mediated graft rejection.

In this study we used a fully MHC-mismatched renal allograft model using mice with targeted deletion of C5aR to explore the hypothesis that C5aR is critical in priming the immune response against donor antigen. We show that deficiency of C5aR prolonged renal allograft survival, which was associated with impaired donor and recipient APC function, impaired recipient T cell responses to allostimulation and reduced cellular infiltration of grafts.

RESULTS

Deficiency of C5aR in Both Graft and Recipient Prolongs Renal Allograft Survival

To determine the role of C5aR in allograft rejection, we initially performed two sets of kidney transplants using C57BL/6 donors and BALB/c recipients: (1) WT donor kidneys into WT recipient (WT to WT) (n = 12); (2) C5aR−/− donor kidneys into C5aR−/− recipients (C5aR−/− to C5aR−/−) (n = 19).

After transplantation, we monitored graft survival for up to 100 days. All grafts in the WT to WT group were lost within 11 days posttransplantation. However, the graft survival rate in the C5aR−/− to C5aR−/− group was significantly increased, in that 47.3% (9/19) of grafts survived for >10 days, 36.8% (7/19) of grafts survived for >30 days, and 15.7% (3/19) of grafts achieved long-term survival (up to 100 days) (Figure 1A, Table 1). A control syngeneic transplant group showed indefinite graft survival up to 100 days posttransplantation (Figure 1A), at which point the recipients were sacrificed. These results indicate that deficiency of C5aR in both graft and recipient prolongs renal allograft survival.

To verify the cause of rejection, we performed histologic analysis of allografts collected at the endpoint of graft survival (with a serum urea of ≥50 mmol/L) from the two experimental groups described thus far. Pathologic changes including vasculitis, cellular infiltration, and tubule damage/tubulitis

Figure 1. Deficiency of C5aR in both graft and recipient prolongs renal allograft survival. Kidney transplant was performed in three groups: (1) WT C57Bl/6 donors and WT BALB/c recipients (WT to WT), (2) C5aR−/− C57Bl/6 donors and C5aR−/− BALB/c recipients (C5aR−/− to C5aR−/−); (3) WT BALB/c donors and WT BALB/c recipients (syngeneic control). Allograft rejection was monitored up to 100 days. (A) Effect of C5aR status on renal allograft survival. Number of animals transplanted per group is included in parentheses. Data were analyzed using the log-rank test (P = 0.0015). (B) Histology of rejecting allografts. Grafts were harvested once serum urea reached ≥50 mmol/L and analyzed by H&E staining of paraffin sections (Magnification: ×100). (C) Mixed lymphocyte reaction. At day 6 after kidney transplantation, splenocytes isolated from C5aR−/− recipients of C5aR−/− kidney allografts or WT recipients of WT kidney allografts were re-stimulated with donor splenocytes. T cell proliferation was measured by 3H-thymidine uptake and IFN-γ secretion was measured by ELISA. Each dot represents a single animal and data are shown as mean ± SEM of replicate wells of the ex vivo culture (n = 4 (for IFN-γ secretion) and n = 8 (for proliferation)). Data were analyzed by t test.
were observed, suggesting that rejection was cell-mediated in both WT and C5aR−/− allografts (Figure 1B).

Because acute rejection is principally T cell–mediated, we next addressed whether an absence of C5aR could influence T cell priming in our model. We examined the proliferative response of primed alloreactive T cells collected from recipients at day 6 post-transplantation (the additional sets of transplants shown in Figure 4) by re-stimulating them ex vivo with splenocytes of donor strain. Both allospecific T cell proliferation and cytokine production were significantly reduced in C5aR−/− recipients of C5aR−/− grafts when compared with those in WT recipients of WT grafts (Figure 1C). Together, these results indicate that the presence of C5aR in both the donor and recipient is required for mounting a full adaptive immune response against donor antigen.

Lack of C5aR on DCs Reduces the Capacity of DCs To Stimulate T Cells

The principal effector mechanism underlying organ transplant rejection is the vigorous adaptive immune response mounted by recipient T cells against donor alloantigen, synthesized and displayed by donor APCs and/or captured, processed, and displayed by recipient APCs.17 To determine if C5aR signaling is critical for donor APC function in T cell stimulation (direct), we investigated whether C5aR−/− DCs from donor strain have a reduced capacity to prime allogeneic T cells from the recipient strain. When compared with DCs from WT donor strain, DCs prepared from C5aR−/− donor strain display a reduced capacity to stimulate allospecific T cells (Figure 2A). To determine whether recipient DC function (indirect) is dependent on C5aR signaling, we used two models: namely, alloantigen presentation or conventional antigen presentation in which specific foreign antigen (ovalbumin [OVA]) is captured, processed by self-APCs, and then presented to OVA-restricted T cells. DCs prepared from C5aR−/− recipient strain, after being loaded with OVA or donor tissue antigen, induced impaired antigen-specific T cell responses, when compared with DCs prepared from WT recipient strain (Figure 2, B and C). These results indicate that the ability of DCs to stimulate T cell activation, in the context of both direct and indirect antigen presentation, is dependent on C5aR signaling.

Lack of C5aR Reduces Recipient T Cell Responses to Allostimulation

The experiments shown above have suggested that C5aR signaling can upregulate allospecific T cell responses through modulation of DC function. To determine whether the presence of C5aR on T cells could have an effect on T cell responses to allostimulation, we prepared naïve T cells from WT mice or C5aR−/− mice and assessed their activity after stimulation by allogeneic DCs. T cells from WT mice demonstrated higher cell proliferation and IFN-γ production when compared with T cells from C5aR−/− mice (Figure 3). This suggests that C5aR signaling in T cells is required for generating a full allospecific T cell response. However, the effect of C5aR expression on T cells appears smaller than the effect observed in DCs.

Effect of C5aR Deficiency on Early Inflammatory Responses within Renal Allografts

Given the role of C5aR in the induction of inflammation and the contribution of initial inflammatory responses within the
Figure 3. Lack of C5aR reduces T cell response to allostimulation. CD4 T cells were prepared from recipient strains (i.e., WT BALB/c and C5aR<sup>−/−</sup> BALB/c) and cocultured with allogeneic WT DCs. T cell responses were assessed by [3H]-thymidine uptake and IFN-γ secretion. Data are shown as mean ± SEM of replicate wells of the ex vivo culture [n = 4 (for IFN-γ secretion) and n = 8 (for proliferation)]. Data were analyzed by t test. ***P < 0.0004; **P < 0.008. A representative of three independent experiments is shown.

Figure 4. C5aR deficiency decreases cellular infiltration within renal allografts. Kidney transplants were performed in two groups: (1) WT C57BL/6 donors and WT BALB/c recipients (WT to WT); (2) C5aR<sup>−/−</sup> C57BL/6 donors and C5aR<sup>−/−</sup> BALB/c recipients (C5aR<sup>−/−</sup> to C5aR<sup>−/−</sup>). Renal allografts were harvested at days 2 and 6 posttransplantation. (A) H&E staining of paraffin sections (magnification ×100). Arrows point to cellular infiltration at the areas around the vessels. (B) Immunohistochemical staining for IFN-γ positive cells. Representative images of three grafts for panels (A) and (B) are shown.

Table 2. Cellular infiltration within renal allografts

<table>
<thead>
<tr>
<th>Cell Types</th>
<th>Cells/Cross Section</th>
<th>WT to WT (n = 3)</th>
<th>C5aR&lt;sup&gt;−/−&lt;/sup&gt; to WT (n = 3)</th>
<th>P</th>
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<tr>
<td>F4/80&lt;sup&gt;+&lt;/sup&gt;</td>
<td>208 ± 14.7</td>
<td>164 ± 8.5</td>
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<tr>
<td>Gr-1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>82 ± 9.6</td>
<td>77 ± 4.4</td>
<td>0.6</td>
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</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>120 ± 11.5</td>
<td>110 ± 8.2</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>IFN-γ&lt;sup&gt;+&lt;/sup&gt;</td>
<td>25.7 ± 5.3</td>
<td>9.3 ± 1.2</td>
<td>0.008</td>
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Lack of C5aR Reduces IFN-γ–Producing Cells and IFN-γ Production in Renal Allografts

In the above experiments, we demonstrated that lack of C5aR reduces allospecific T cell responses ex vivo, but has no apparent effect on T cell infiltrates in the day 6 grafts. To pursue this further, we investigated whether functional activity of T cells within the allografts was influenced by C5aR. We performed reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical staining for IFN-γ in day 6 allografts (i.e., WT to WT and C5aR<sup>−/−</sup> to C5aR<sup>−/−</sup>). Our RT-PCR results showed that mRNA transcripts for IFN-γ were elevated in both allograft groups compared with normal kidney tissue. However, WT to WT allografts exhibited a higher level of IFN-γ expression than C5aR<sup>−/−</sup> to C5aR<sup>−/−</sup> allografts (Figure 5A). Consistent with the RT-PCR results, immunohistochemical stain-
ing showed that the number of IFN-γ–producing cells is higher in WT to WT allografts than in C5aR−/− to C5aR−/− allografts at day 6 posttransplantation, and the majority of IFN-γ–positive cells exhibit lymphocyte morphology, particularly in WT-WT grafts (Figure 5B). Although other immune cells such as activated macrophages within the grafts could be a source of IFN-γ, considering the fact that activated T cells are the major source of IFN-γ once antigen-specific immunity develops, our results suggest T cell activity in WT to WT transplants is higher than that in the C5aR−/− to C5aR−/− group.

Effect of C5aR Deficiency in Graft or Recipient Alone on Renal Allograft Rejection

In Figure 1, we have demonstrated a clear association between increased allograft survival and deficiency of C5aR in both graft and recipient. To determine the contribution of either donor C5aR or recipient C5aR expression to renal allograft rejection, we performed two additional sets of transplants using the same strain combination: (1) C5aR−/− to WT (n = 12); (2) WT to C5aR−/− (n = 8). For ease of comparison, allograft survival rates are presented alongside graft survival rates of WT-WT transplants shown in Figure 1. Unexpectedly, all allografts in both C5aR−/− to WT and WT to C5aR−/− groups were rejected within 11 days posttransplantation. Graft survival rates were not significantly different when compared with those of the WT to WT group (Figure 6). This result indicates that C5aR deficiency in either graft or recipient alone is not sufficient to prevent renal allograft rejection in our model.

We therefore used a less aggressive model of rejection, treating recipients with a course of rapamycin, an immunosuppressant drug that reduces the rate of renal allograft rejection,18–20 to detect any subtle effect of donor or recipient C5aR on graft rejection. We performed three sets of transplants using C57BL/6 kidney donors and BALB/c recipients: (1) WT to C5aR−/− (n = 5); (2) C5aR−/− to WT (n = 7); (3) WT to WT (n = 5). Recipient mice received 7.5 μg of rapamycin intraperitoneally for 3 consecutive days, commencing the day of Figure 5. Lack of C5aR reduces IFN-γ–producing cells and IFN-γ mRNA expression in renal allografts. Kidney transplants were performed in two groups: (1) WT C57B/6 donors and WT BALB/c recipients (WT to WT); (2) C5aR−/− C57B/6 donors and C5aR−/− BALB/c recipients (C5aR−/− to C5aR−/−). Renal allografts were harvested at day 6 posttransplantation. (A) RT-PCR of renal tissue (i.e., normal and allografts) for IFN-γ. Data are shown as mean ± SEM of duplicate PCR of three grafts or normal kidneys (n = 6). (B) Immunohistochemical staining of frozen sections for IFN-γ. (a, b) The areas around the vessel. (c, d) The areas of renal tubules. Representative image of three grafts for each group is shown.

Figure 6. C5aR deficiency in either graft or recipient alone is not sufficient to prevent renal allograft rejection. Kidney transplants were performed in two groups: (1) C5aR−/− C57B/6 donors and WT BALB/c recipients (C5aR−/− to WT); (2) WT C57B/6 donors and C5aR−/− BALB/c recipients (WT to C5aR−/−). For ease of comparison, the graft survival rates in these two groups were compared with survival rates in WT-WT transplants shown in Figure 1. Number of animals transplanted per group is included in parentheses. Data were analyzed using the log-rank test. (A) C5aR−/− to WT transplants versus WT to WT transplants. (B) WT to C5aR−/− transplants versus WT to WT transplants.
transplantation. Graft survival was then monitored for up to 100 days. Rapamycin treatment prolonged graft survival in all three groups when compared with those recipients that received no rapamycin in previous experiments. Interestingly, with rapamycin treatment, graft survival was extended in the WT to C5aR−/− group (median survival time (MGT) = 100 days) when compared with survival in the WT to WT group (MGT = 13 days). However, graft survival in the C5aR−/− to WT group (MGT = 24 days) was not significantly different from that in the WT to WT group (MGT = 13 days) (Figure 7). This observation clearly indicates that C5R deficiency in the rapamycin-treated recipient (but not the donor) conferred protection from allograft rejection in our model.

**DISCUSSION**

Recent research has shown that several innate immune receptors (e.g., toll-like receptors and chemokine receptors) are involved in allograft rejection (reviewed by21,22). In particular, it has been shown that treatment of recipient mice with a C5aR antagonist significantly improved renal allograft survival,23 suggesting a role for C5aR in allograft rejection.

However, it is unclear whether the effect of C5aR on kidney transplant rejection occurs through an effect upon the innate immune response or the C5aR-dependent mechanism modulates cognate immune recognition of donor antigen, and in particular whether C5aR modulates antigen-presenting cell priming. This is an important mechanism to address as a growing body of evidence implicates complement activation as a coactivator that enhances the adaptive immune response.2,24,25 In this study, the use of receptor-knockout mice allowed us to define the role of C5aR in renal allograft rejection in a more specific manner, as the receptor antagonist may act on other receptors (e.g., C5L2). The knockout mice also enabled us to investigate the specific effect of C5aR deficiency in the graft or recipient alone on renal allograft rejection, and on donor and recipient APC function. Thus, this study not only provides definitive evidence supporting a role for C5aR in promoting renal allograft rejection but also identifies several possible processes for graft rejection modulated by C5aR signaling. These include both donor and recipient DC function in T cell stimulation, T cell alloreactivity, and cellular infiltration of renal allografts.

An important question arising from our observations is how C5aR signaling contributes to renal allograft rejection. Allorecognition involves both APCs and T cells occurring through two, nonmutually exclusive pathways (direct and indirect allorecognition). In the direct pathway, allospecific T cells recognize intact donor MHC molecules on APCs that are present in the transplanted organ. In contrast, through the indirect pathway, host APCs take up and process Ag derived from donor MHC molecules and present them to alloreactive T cells in a self-restricted manner.17 Therefore, the activation state of donor APC and the function of host APC in antigen presentation, and also host T cell alloreactivity, can influence the efficiency of allorecognition. Given that C5aR is expressed on both DCS and T cells11,14–16 and C5a are present in the circulation and can be generated in the local milieu in response to inflammation26 and be detected in cocultures of APC and T cells,11 it is therefore possible that C5a engages C5aR on both donor and recipient DCs leading to cell activation, with a consequent upregulation of donor DC’s allostimulatory capacity and recipient DC’s function in antigen presentation. Additionally, engagement of C5aR on T cells may cause T cell activation, thereby enhancing T cell alloresponses to allostimulation. Observations made in this study strongly support this explanation. First, donor DCs prepared from C5aR−/− mice displayed reduced capacity to stimulate allospecific T cells. Inconsistent with this observation, we found that DCs prepared from C5aR−/− mice exhibited a less activated phenotype, with reduced surface expression of both MHC Class II and B7.2, compared with WT mice.27 Second, recipient DCs prepared from C5aR−/− mice, after being loaded with foreign antigen or alloantigen, induced an impaired antigen-specific T cell response. Furthermore, T cells prepared from C5aR−/− mice displayed reduced T cell reactivity in response to stimulation by allogeneic DCs. Thus, C5aR signaling–mediated upregulation of both donor and recipient DC’s function in T cell stimulation and also T cell activation could contribute to the enhanced adaptive alloimmune response with consequent renal allograft rejection.

In addition to upregulating adaptive alloimmune responses, C5aR signaling may also modulate the innate immune response. Previous studies have shown that C5aR signaling contributes to renal ischemia/reperfusion injury, which has a major effect on renal allograft rejection.7,8,28 In this study, we explored the possibility that C5aR signaling increases early allograft inflammation, thus contributing to renal allograft rejection. This is an important question to address, as the innate response.
immune response to tissue injury is considered to be closely associated with allotransplantation. C5αR is a potent leukocyte chemoattractant receptor and previous research has suggested that infiltrating leukocytes, for example, neutrophils and monocytes/macrophages, can mediate tissue damage through an array of cytotoxic and proinflammatory mechanisms that include the release of proinflammatory cytokines, generation of reactive nitrogen and oxygen products, and presentation of antigen to effector T cells. We hypothesized that C5αR signaling can modulate leukocyte infiltration of the allograft, thereby contributing to graft rejection. Indeed, our results show that deficiency of C5αR in both graft and recipient diminishes early inflammation in the grafts, with a lesser degree of cellular infiltration around the vessels and reduced numbers of F4/80–positive cells in the peritubular interstitium. These findings suggest that C5αR signaling is important for cellular infiltration and macrophage accumulation within allografts. Cellular infiltration is a major feature of acute renal allograft rejection and macrophages, which constitute 40 to 60% of infiltrating cells during acute renal allograft rejection, which can participate in both innate and adaptive immune responses. Thus, our data support the notion that C5αR signaling contributes to renal allograft rejection by modulating cellular infiltration and macrophage accumulation within the grafts. The mechanisms by which C5α/C5αR interaction induces monocyte/macrophage infiltration/accumulation within the grafts remain to be clarified. Because T cell priming orchestrates the effector response, a reduced accumulation of macrophages is likely to result from, at least in part, reduced T cell priming in the absence of C5αR. However, our own unpublished observations show that after peritoneal injection of inflammatory stimuli, macrophage recruitment into the peritoneum is impaired in C5αR−/− mice and the migrating macrophages exhibited a reduced activation phenotype, indicating an important antigen-independent effect of C5αR on macrophage function. Previous research also suggests that C5α may directly or indirectly influence the recruitment and activation of monocytes/macrophages by upregulating other chemotaxtrant factors such as MCP-1 and ICAM-1.

We investigated the relative contribution of C5αR signaling in the graft and the recipient to allograft rejection. C5αR is expressed on a range of myeloid cells and parenchymal cells (e.g., T cells, DCs, neutrophils, macrophages, basophilic leukocytes, mast cells, endothelial cells, and epithelial cells). Several of these cell types are also present in donor tissues, including macrophages and DCs (review by 21). Our in vitro data clearly demonstrated that, in the absence of C5αR, the capacity of both donor and recipient DCs to stimulate T cells was reduced, with a concurrent decrease in recipient T cell activation. Our in vivo data showed that lack of C5αR reduced macrophage accumulation, for which both donor and recipient macrophages could account for. Together, our data suggests that both donor and recipient C5αR signaling could contribute to allograft rejection. We therefore explored the possibility that deficiency of either graft or recipient C5αR alone prolongs renal allograft survival. Our data clearly demonstrates that a combined absence of recipient and donor C5αR signaling is capable of prolonging renal allograft survival. This observation suggests that both donor and recipient C5αR signaling can contribute to the alloimmune response and allograft rejection. However, the presence of C5αR in the donor or recipient alone is not sufficient to prolong allograft survival. Our data showing that absence of recipient C5αR alone combined with rapamycin treatment prolonged graft survival, together with the influence of C5αR signaling on several cellular processes that involve the recipient (e.g., APC function, T cell activation, and cellular infiltration), suggest that recipient C5αR singling is relatively more important than donor C5αR singling in promoting allograft rejection in our model. It is possible that the less aggressive rejection associated with rapamycin treatment unmasked a more subtle effect of recipient-expressed receptor.

In conclusion, in this study we demonstrate that C5αR deficiency resulted in prolonged graft survival and reduced graft infiltration by macrophages. We also show a reduction of T cell priming in the absence of C5αR on donor/recipient APCs and recipient T cells. It is therefore likely that our current observation of prolonged graft survival in C5αR-deficient transplantation is at least in part explained by both deficient T cell priming and early inflammatory responses within the graft. These findings provide a better understanding of how C5αR participates in the regulation of adaptive alloimmunity. In addition, our data suggest that targeting C5α-mediated signaling to prevent graft injury may require inhibition of both the donor and the recipient C5αR pool. To provide definitive and detailed explanations for the prolonged graft survival in C5αR-deficient transplantation, more studies are required. We speculate that C5α-C5αR-mediated monocyte/macrophage infiltration of the allograft may involve more elements of immune responses such as alloantibody responses and regulatory T cell development. In addition, engagement of C5αR on other cells (e.g., renal microvascular endothelial cells, mast cells, and neutrophils) may lead to cell functional modulation, thereby contributing to allograft rejection.

**CONCISE METHODS**

**Reagents** Recombinant murine GM-CSF was purchased from R&D Systems Europe Ltd. (Abingdon, United Kingdom). CD11c micro bead and FITC-conjugated anti-mouse CD11c antibody were purchased from Miltenyi Biotec UK (Surrey, United Kingdom). Antibody for CD3 was purchased from Cambridge BioScience Ltd. (Cambridge, United Kingdom). The antibodies for Gr-1 and F4/80 were purchased from Serotec (Oxford, United Kingdom). The antibody for INF-γ was purchased from Insight Biotechnology Ltd. (London, United Kingdom). HRP-conjugated rabbit anti-rat polyclonal antibody was purchased from DAKO (Cambridge, United Kingdom). Spin-Sep Enrichment Cocktail kit used for CD4 T cell purification was purchased from Stem Cell Technologies UK (London, United Kingdom). ELISA kits used...
for measuring IFN-γ were purchased from BD Biosciences (Cowley, United Kingdom). 3H-Thymidine was purchased from GE Healthcare UK Ltd. (Bucks, United Kingdom).

Mice
Homozygous C5aR−/− mice were derived by homologous recombination in embryonic stem cells35 and backcrossed on to the C57BL/6 (H-2b) or BALB/c (H-2d) parental strain for at least 12 generations. Male mice (6 to 8 weeks old) were used in all experiments and animal procedures were carried out within the Animals (Scientific Procedures) Act 1986.

Renal Transplantation
Renal transplantation was performed using C57BL/6 donors and BALB/c recipients. The technique was adapted from a previously published method.36,37 Donor mice were anesthetized and the abdomen was opened via 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. Urinary tract reconstruction was accomplished using ureter-to-bladder anastomosis. No immunosuppressive therapy was administered at any time during the experiment, unless otherwise specified. The left native kidney was removed at 1 week posttransplantation. The graft was placed in the right flank as left nephrectomy is technically easier to perform. The endpoint of graft survival was defined by blood urea nitrogen >50 mmol/L or death of the animal, depending on which was first.

Kidney Histology
Kidneys were fixed in 4% paraformaldehyde (pH 7.4) and embedded in paraffin. Paraffin sections (2 μm) were air-dried and then acetone-fixed. Kidneys were fixed in 4% paraformaldehyde (pH 7.4) and embedded in paraffin. Paraffin sections (2 μm) were air-dried and then acetone-fixed. Immunohistochemical staining was performed on frozen kidney tissue. Frozen sections (4 μm) were air-dried and then acetone-fixed. Indirect immunohistochemical staining for T cells, macrophages, neutrophils, and IFN-γ was performed using rat monoclonal anti-CD3, Gr-1, or F4/80 antibody or IFN-γ (all at 1:50 dilution) and HRP-conjugated rabbit anti-rat polyclonal antibody. Positively stained cells were quantified at a magnification of ×200 and 10 fields from each stained section and expressed as cell numbers per cross section.

Preparation of DCs
DCs were cultured from bone marrow (BM) progenitor cells as described previously with minor modifications.38 Briefly, BM cells were isolated from mouse femurs and tibias and cultured in DC medium (RPMI 1640 containing 5% FCS, 50 μM β-mercaptoethanol, 100 μg/ml streptomycin, and 20 ng/ml GM-CSF). Culture medium was replaced every other day, and on day 6, DCs were collected and enriched with CD11c microbeads. The purity of the DC preparation was routinely >80%, as determined by flow cytometry analysis.

Mixed Lymphocyte Reaction
Splenocytes (2 × 10^7) derived from BALB/c mice that had received a C57BL/6 kidney were cocultured with irradiated (2000 Rad) allogeneic splenocytes (2 × 10^7) in T cell culture medium (RPMI 1640 containing 10% FCS, 50 μM β-mercaptoethanol, 50 μg/ml gentamicin, and 2.5 μg/ml fungizone). 3H-Thymidine uptake was performed after 3 days of coculture. IFN-γ secretion was measured by ELISA after 5 days of coculture.

Analysis of Donor DC’s Capacity To Stimulate Alloreactive T Cells
The assay for assessing DC function in allostimulation was performed as described previously.14 Briefly, irradiated C57BL/6 DCs (2 × 10^4 per well) were cocultured with BALB/c CD4 T cells (2 × 10^5 per well) in T cell culture medium. 3H-Thymidine uptake was performed after 3 days of coculture. IFN-γ secretion was measured by ELISA after 5 days of coculture.

Analysis of Recipient DC’s Function in Antigen Presentation
The assay for assessing DC’s function in antigen presentation was performed in two models (OVA, alloantigen) as described previously.39,40 For OVA presentation, BALB/c DCs (2 × 10^4 per well), after being incubated with OVA protein (250 μg/ml) at 37°C for 40 minutes, were cocultured with OVA-specific CD4 T cells (from DO11.10 mice with BALB/c background) (2 × 10^5 per well) in T cell culture medium. For alloantigen presentation, BALB/c DCs (4 × 10^4 per well), after being incubated with C57BL/6 tissue antigen (5 μg/ml) at 37°C for 20 hours, were cocultured with BALB/c CD4 T cells (2 × 10^5 per well) in T cell culture medium. 3H-Thymidine uptake was performed after 3 or/and 6 days of coculture. IFN-γ secretion was measured by ELISA after 5 days of coculture. The method for preparation of tissue antigen is shown in the Supplemental Text.

Quantitative RT-PCR
Quantitative RT-PCR was performed using an MJ Research PTC-200 Peltier Thermal Cycler and DyNAmo Green qPCR kit (MJ BioWorks, Finland). PCR was set up in 96-well microplates containing 10 μl of master mix, 2 μl of cDNA (reflecting 0.2 μg of total RNA), and 10 pmol each of 3′ and 5′ primer pair, either for the IFN-γ gene or ribosomal RNA 18S (18S), in a 20-μl reaction volume. Amplification was performed according to manufacturer’s cycling protocol and tested in duplicate. Gene expression was calculated as 2−ΔΔ(Ct), where Ct is cycle threshold, ∆(Ct) = 18S(Ct) − testing gene(Ct), and ∆Δ(Ct) = sample 1 ∆(Ct) − sample 2 ∆(Ct). The PCR primer sequences and product sizes are shown in the Supplemental Text.

Statistical Analysis
ELISA was performed in four replicate coculture wells. T cell proliferation assays were performed in six to eight replicate coculture wells. Results were expressed as arithmetic means (±SEM). Statistical analyses between different experimental groups were performed using unpaired t test or one-way ANOVA, and survival analysis was calculated using the log-rank method. A difference was considered signif-
significant when $P < 0.05$. All of the analyses were performed using GraphPad Prism Software.

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DISCLOSURES

None.

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