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Donor specific transplant tolerance is dependent on complement receptors

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Summary

The complement system has recently been described as a crucial component for transplant tolerance induction, but the underlying mechanisms are poorly understood. Using a rodent model of donor lymphocyte infusion-induced male histocompatibility antigen-specific transplant tolerance, we demonstrate that tolerance induction is dependent on the complement receptors decay accelerating factor, complement receptor 3, and complement component 3a receptor (C3aR). Furthermore, we have provided evidence that complement dependent tolerance is mediated through C3aR on infused donor splenocytes and on recipient cells. *Ex vivo* studies showed that C3aR deficiency leads to an imbalance between T regulatory and T effector cells. Increased numbers of antigen-specific $CD8^+$ cells in the blood and less T regulatory cells, with reduced suppressive function, in the spleen and in the skin grafts were detected in C3aR deficient compared to wild type mice. This imbalance might be explained by the requirement of complement for dendritic cells to generate T regulatory cells effectively. Our experiments suggest that multiple complement receptors play an important role in transplant tolerance induction providing new insights into the mechanisms of complement dependent tolerance.

Introduction

Transplantation is the treatment of choice for patients with end-stage organ failure. Immune-mediated organ rejection is the main cause of graft failure, and is currently prevented by immunosuppressive drugs, which predispose patients to infection and malignancy. Achieving donor specific tolerance would eliminate these shortcomings. A number of tolerance induction protocols have been successfully applied in animal models [1]. However, the same strategies have yielded limited success in humans [2]. Improved understanding of the mechanisms by which these tolerogenic protocols exert their effects may aid the development of effective clinical protocols.

The complement system is part of the innate immune system and orchestrates innate and adaptive immune response. It has been documented most extensively in its destructive capacity. However, there is growing evidence that the complement system is also important to control the immune response [3,4]. Moreover, it has been shown that complement component 3 (C3) split products are important in the induction and maintenance of tolerance [5–7]. This effect is mediated via the complement split product iC3b binding on complement receptor 3 (CR3) on antigen presenting cells (APC). Recent publications have demonstrated that C3 and complement component 1q are crucial for male histocompatibility antigen Y (HY) specific allograft tolerance induction [8,9], and complement deficient animals showed an increase in T effector cells (Teff). However, the underlining mechanisms for this complement dependent transplant tolerance remain unknown. As complement inhibition is a potentially novel therapeutic strategy for recipient desensitization [10] and prolonging renal allograft survival [11], it is

important to ascertain whether complement plays a role in protocols used for the induction of transplant tolerance and to identify the precise products of complement activation that are responsible.

To unravel the mechanisms by which the complement system contributes to transplant tolerance induction, we used a rodent model of complement dependent tolerance. In contrast to wild type (WT) animals, it is not possible to induce tolerance in female recipients of a male skin graft and a donor lymphocyte infusion (DLI) in the absence of C3 [8]. Using complement receptor deficient mice, we demonstrated that complement dependent tolerance is dependent on a specific set of complement receptors *in vivo*. In addition, we further characterized the subsets of lymphocytes that are involved in complement dependent tolerance *in vitro* and provide mechanisms by which the complement system orchestrates these cells toward tolerance induction.

Materials and methods

Mice

Animals were kept in specific pathogen-free animal facilities and were used between the age of 8 and 12 weeks in accordance with the Animals (Scientific Procedures) Act 1986. C57BL/6 (H-2^b), BALB/c (H-2^d), and CBA (H-2^k) mice were purchased from Harlan Limited (Indianapolis, IN, USA) and Charles River (Wilmington, MA, USA), respectively. Homozygous $CR3^{-/-}$ ($CD11b^{-/-}$) C57BL/6 mice were kind gifts from Dr. Bao Lu (Harvard Medical School, USA). Homozygous $C3^{-/-}$ C57BL/6 and complement component 3a receptor $(C3aR)^{-/-}$ C57BL/6 mice were kind gifts from Drs. M Carroll and Bao Lu, respectively (Harvard Medical School). Decay accelerating factor $(DAF)^{-/-}$ C57BL/6 mice were kind gifts from Dr. BP Morgan (Cardiff University, School of Medicine, UK).

Skin transplantation

Skin transplantation was performed as previously described [12], except full thickness trunk skin, was used instead of tail skin. Rejection was defined as >90% necrosis of the grafted skin.

Donor lymphocyte infusion protocol

Male spleens were harvested, passed through a 40 μm cell strainer and washed in phosphate buffered saline (Oxoid, Hampshire, UK). Pharm lyse (Pharmingen, Franklin Lakes, NY, CA, USA) was used according to manufacturer's instruction. After washing cells in phosphate buffered saline, 35 million cells were injected intravenously immediately before skin transplantation.

Flow cytometry

Cell-surface molecules were stained with fluoresceine isothiocyanate (FITC) conjugated rat anti-mouse CD4 (clone GK 1.5, Pharmingen), phycoerytrin (PE) conjugated rat anti-mouse CD25 (clone 7D4, Myltenyi, Bergisch-Gladbach, Germany) peridinin chlorophyll protein (PerCP) 5.5 conjugated rat anti-mouse CD8 (clone 53–6.7, Pharmingen), PE conjugated HY specific ubiquitously transcribed tetratricopeptide repeat gene, Y-linked D^b tetramer (allele, H-2 D^b/PE; peptide, WMHHNMDLI, Beckman Coulter, Bra, CA, USA), allophycocyanin rat anti-mouse CD45R/B220 (clone RA3-6B2, Pharmingen), PE rat anti-mouse CD38 (clone 90/CD38, Pharmingen), FITC rat anti-mouse IgM (clone R6-60, Pharmingen), PE/cyanine 7 rat anti-mouse CD11c (clone N418, BioLegend, San Diego, CA, USA), biotinylated rat anti-mouse IA^b antibody (clone AF6-120.1, Pharmingen), and strepatvidin FITC (Pharmingen). Intracellular staining of forkhead box P3 (FoxP3) was performed using biotinylated rat anti-mouse/rat FoxP3 (clone FJK-16s, eBioscience, San Diego, CA, USA) and streptavidin PerCP 5.5 (eBioscience). Cells were acquired using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NY, USA) and analyzed using CellQuest Pro (BD Biosciences) and Win MDI 2.9 Software (Joseph Trotter).

In vitro dendritic cell generation

Dendritic cells (DC) were generated as previously described [13] with the following modifications. B-, T-, and MHC class II positive cells were depleted using monoclonal mouse anti-mouse IA^b (clone 25-9-17, BioLegend), monoclonal rat anti-mouse CD45R/B220 (clone RA3-6B2, BioLegend), monoclonal rat anti-mouse CD4 (clone YTS 3.1, grown in house from hybridoma), and monoclonal rat anti-mouse CD8a (clone YTS 169, Cedarlane, Burlington, Ontario, Canada). Enriched DC precursor cells were supplemented with 4 ng/ml of mouse recombinant granulocyte macrophage colony-stimulating factor (PreproTech, Rocky Hill, NJ, USA) and 2 ng/ml of human recombinant transforming growth factor beta isoform 1 (PreproTech) and 0.5 mM 2-Mercaptoethanol (Sigma, St. Louis, MO, USA).

In vitro T regulatory cell generation

T regulatory cells (Treg) were generated *in vitro* as previously described [14] with the following modifications. 5×10^5 $CD4^+$ cells were isolated from splenocytes with magnetic beads (L3T4 MicroBeads, Miltenyi) used according to the manufacturer's instructions and co-cultured with 5×10^5 bone marrow derived DC in 24-well plates in the

presence of 10 ng/ml mouse recombinant interferon gamma (IFN-γ; PreproTech) and 0.5 mM 2-Mercaptoethanol (Sigma).

Suppression assay

$CD4^+CD25^-$ and $CD4^+CD25^+$ cells were harvested from spleen using magnetic beads ($CD4^+CD25^+$ Regulatory T Cell Isolation Kit, mouse, Miltenyi) according to the manufacturer's protocol. Feeder splenocytes from BALB/c spleen were irradiated with 3 000 radiation-absorbed doses after removing red blood cells using an ammonium chloride-based lysing reagent (BD Pharm Lyse, Pharmingen) according to the manufacturer's instructions. $CD4^+CD25^-$ cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA, USA) used according to the manufacturer's instructions. Co-cultures were set up with 1×10^4 CFSE labeled $CD4^+CD25^-$ cells with $CD4^+CD25^+$ cells at ratios of 1:1, 1:0.5, 1:0.25, 1:0.125 and 1:0, respectively, in the presence of 3×10^5 feeder splenocytes and 0.5 μg/ml soluble NA/LE hamster anti mouse-CD3e (clone 145-2C11, Pharmingen) in 96 U-bottom well plates and filled with RPMI 1640 supplemented with 2 mM L-glutamine (Sigma), 10% fetal calf serum (Invitrogen), 0.1 mM 2-Mercaptoethanol (Sigma), and 100 U/ml each of penicillin and streptomycin (Sigma). Proliferation index (PI) of CFSE-labeled CD^+CD25^- cells was calculated at day 4 according to the following formula: all cells/(non proliferating cells + F1 generation/2+ F2 generation/4+ F3 generation/8+ F4 generation/16+ F5 generation/32).

Immunohistochemistry

For immunohistochemistry, standard methods were used [15]. Frozen skin and skin grafts were cut into 8 μm sections. Staining for CD8, CD4, FoxP3 surface antigens was performed using a modified previously described protocol [15]. Tissue was fixed in acetone/methanol (1:1) and endogenous peroxidase activity within the tissue was quenched with 3% hydrogen peroxide in methanol. Foxp3 was identified using a biotinylated rat anti-mouse/rat (clone FJK-16s; eBioscience); CD4 and CD8 were identified using anti-CD4 (clone H129.19, Pharmingen) and anti-CD8 (clone 53-6.7, Pharmingen) rat anti-mouse. For detection biotinylated goat, anti-rat (Pharmingen) was used. Slides were analyzed using a Diaplan microscope (Leitz, Stuttgart, Germany) and a DXM1200DF digital camera (Nikon, Tokyo, Japan), using Lucia G software (Nikon). Positive cells were counted in 20 random high power fields (×400) of each sample by an observer blinded to the experimental conditions. Numbers were displayed as positive cells per mm^2.

Statistical analysis

Kaplan–Meier analysis was applied to calculate skin graft survival. Cell numbers and PI were displayed as median. Error bars indicate a confidence interval of 95%. The Mann–Whitney U Test was used to compare cell numbers and PI between groups. All tests were two-sided, with a 5% type I error. Statistical calculations were performed using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Donor lymphocyte induced transplant tolerance is dependent on C3

Complement component 3 dependent transplant tolerance in the HY skin transplant model has previously been described [8,9,16]. This observation is confirmed using independent experiments here, comparing graft survival in $C3^{-/-}$ and WT mice following DLI induced transplant tolerance (Fig. 1a). We then went on to investigate the mechanisms involved in this model of complement dependent tolerance.

Impact of C3 on IFN-gamma induced Treg generation

The outcome of an immune response is thought to be the result of the balance between Treg and Teff [17]. Defective generation of Treg in the absence of C3 would explain the above described observation. Therefore, we investigated whether generation of Treg is impaired in the absence of C3 in an *in vitro* model of Treg generation co-culturing bone marrow derived DC and $CD4^+$ splenocytes in the presence of exogenous IFN-gamma.

Using male C57BL/6 WT mice as the source of bone marrow and female C57BL/6 WT mice as the source of $CD4^+$ cells, 5.7% $CD4^+CD25^+FoxP3^+$ cells could be generated (Fig. 2). To test whether C3 is crucial for the generation of Treg by DC, we co-cultured male DC with female $CD4^+$ cells, both derived from $C3^{-/-}$ mice. In this C3 free environment, only 2.1% $CD4^+CD25^+FoxP3^+$ cells could be generated, which was significantly less compared with co-cultures using cells derived from WT mice ($P = 0.016$). There was no difference in $CD4^+CD25^+FoxP3^-$ cells in a C3 free environment (data not shown). This suggests that the DC ability to generate IFN-gamma induced Treg is diminished in a C3 free environment. To determine which source of C3 is crucial for the Treg generation, we prepared mixed co-cultures, where DC from $C3^{-/-}$ male mice with $CD4^+$ cells from female WT mice and DC from WT male mice with $CD4^+$ cells from female $C3^{-/-}$ mice. A similar amount of $CD4^+CD25^+FoxP3^+$ cells in mixed co-cultures with WT DC and a lower amount in mixed co-cultures with $C3^{-/-}$ DC compared with complete WT co-cultures was

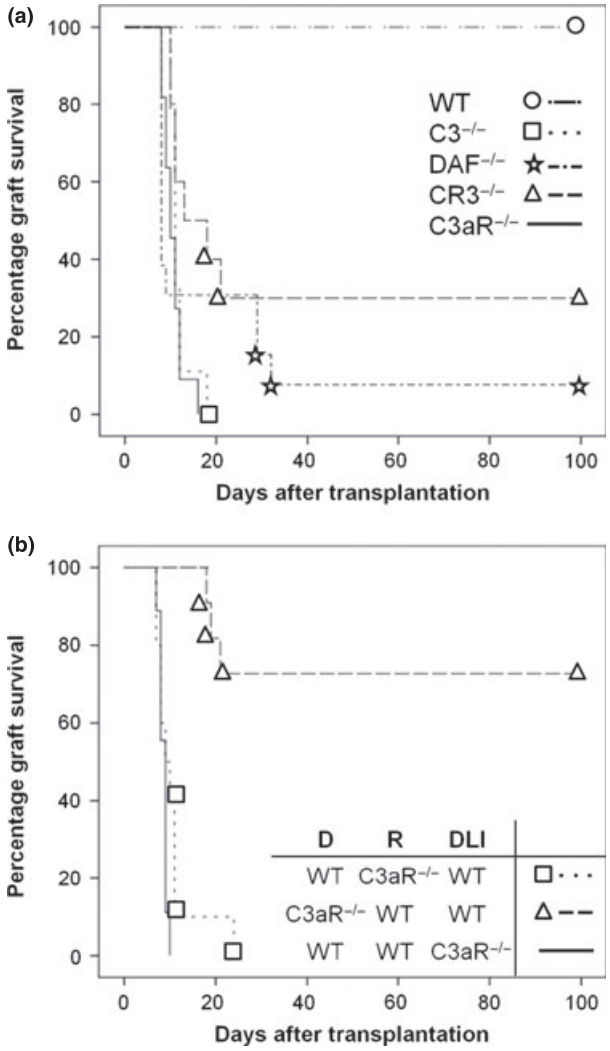


Figure 1 Survival function of skin grafts after tolerance induction. Female recipients received male trunk skin transplants after infusion of 35×10^6 donor splenocytes. (a) WT mice (circle) experienced long-term graft survival (>day 100 after transplantation), whereas in the absence of $C3^{-/-}$ (square) skin grafts were rejected with a MST of 11 days after transplantation ($N = 6$ and $N = 10$ respectively; $P = 0.001$). Likewise, tolerance could not be established in the absence of $DAF^{-/-}$ (star; $N = 12$), $CR3^{-/-}$ (triangle; $N = 10$) and $C3aR^{-/-}$ ($N = 10$), respectively. $DAF^{-/-}$, $CR3^{-/-}$, and $C3aR^{-/-}$ recipients rejected their graft similar to $C3^{-/-}$ mice with a MST of 8 ($P = 0.9$), 16 ($P = 0.05$), and 11 ($P = 0.3$) days after transplantation. (b) Whereas WT recipients of $C3aR^{-/-}$ skin grafts after WT DLI (triangle) experienced long-term graft survival comparable to recipients of a WT skin graft ($N = 11$; $P = 0.18$), $C3aR^{-/-}$ recipients of WT skin (square), and WT DLI and WT recipients of WT skin and $C3aR^{-/-}$ DLI rejected their skin grafts at a similar time compared to complete absence of C3aR (MST 10 [$N = 10$] and 9 [$N = 9$] vs. 11; $P = 0.67$ and $P = 0.13$ respectively). D Donor; R Recipient.

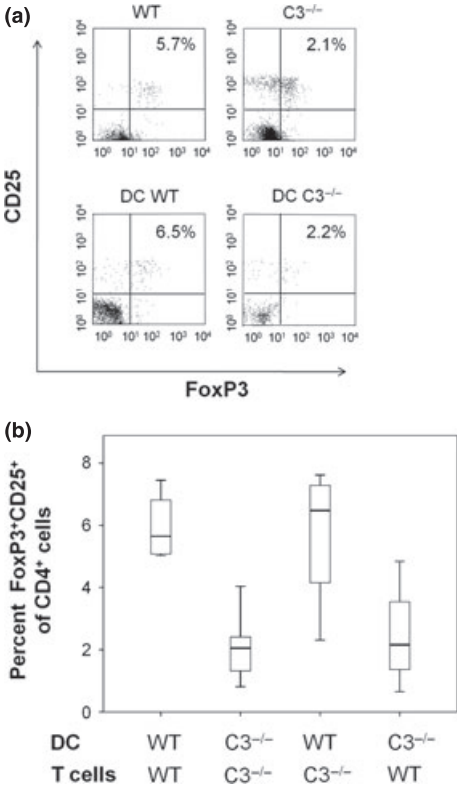


Figure 2 Analysis of IFN-gamma mediated DC induced generation of Treg. $CD4^+$ female splenocytes were co-cultured with bone marrow derived male DC in the presence of IFN-gamma and analyzed at day 14 by FACS. (a) FITC conjugated anti CD4 positive cells were gated on a FL1 histogram and analyzed in a FL2/FL3 dot plot for PE conjugated anti CD25 (FL2) and PerCP 5.5 FoxP3 (FL3) double positive cells. Dot plots show representative examples from WT (left) and $C3^{-/-}$ (right) co-cultures (upper row) and mixed co-cultures (lower row) with WT DC and $C3^{-/-}$ T cells (left) and $C3^{-/-}$ DC and WT T cells (right). Percentages are median numbers analyzed from a minimum of four independent experiments. (b) Significantly, less $FoxP3^+CD25^+$ cells were detectable in a C3 free environment compared to numbers obtained from WT co-cultures (2.1% vs. 5.7%; $P = 0.016$). Whereas mixed co-cultures with DC derived from WT mice and $CD4^+$ T cells from $C3^{-/-}$ mice were able to generate similar amounts of Treg compared to co-cultures with WT DC and WT $CD4^+$ T cells (6.5% vs. 5.7%; $P = 0.89$), co-cultures with DC from $C3^{-/-}$ mice and WT $CD4^+$ T cells generate significantly less Treg compared to co-cultures with WT DC and WT $CD4^+$ T cells (2.2% vs. 5.7%; $P = 0.029$). Error bars indicate a confidence interval of 95%.

measured (6.5% $CD4^+CD25^+FoxP3^+$; $P = 0.89$ and 2.2% $CD4^+CD25^+FoxP3^+$; $P = 0.029$, respectively), i.e. reconstitution with WT DC appeared sufficient to restore the production of Treg in these experiments. These data suggest that C3 produced by DC is enhancing IFN-gamma mediated Treg generation *in vitro*.

DAF is required for complement dependent transplant tolerance

To further dissect the mechanisms of C3 dependent tolerance, we investigated whether this phenomenon is

mediated through receptors that are known to bind C3 *in vivo*. As DAF has been shown to regulate T cell alloreactivity in other animal transplant models [18–20], male to female skin transplants after DLI were performed in $DAF^{-/-}$ animals. Ninety-two percent of $DAF^{-/-}$ recipients rejected their skin grafts with a median survival time (MST) of 8 days (Fig. 1a) in a similar tempo compared to $C3^{-/-}$ mice (MST 11 days; $P = 0.9$), suggesting that DAF is required for DLI induced complement mediated tolerance.

CR3 is required for complement dependent transplant tolerance

Next, we examined whether DLI induced complement dependent transplant tolerance is mediated through CR3, which has been described as crucial in other models of tolerance. It has been shown that CR3 is necessary for the maintenance of tolerance in the anterior chamber of the eye and for ultraviolet induction of immunosuppression in the skin [5,6]. $CR3^{-/-}$ female recipients received skin grafts and DLI from $CR3^{-/-}$ male donors (Fig. 1a). Seventy percent of the $CR3^{-/-}$ rejected their grafts in a slightly delayed manner compared with $C3^{-/-}$ mice (MST of 16 vs. 11 days; $P = 0.05$), suggesting that CR3 is important for DLI induced complement mediated tolerance, but complement factors other than CR3 may also be involved.

C3aR is required for complement dependent transplant tolerance

Signaling through C3aR has been shown to mediate profound effects directly on $CD4^+$ T cells [21] and indirectly via APC [22,23]. We hypothesized that direct or indirect action of complement via C3aR on recipient T cells or donor APC is important for the development of HY specific tolerance in our model. $C3aR^{-/-}$ female mice received skin and DLI from male $C3aR^{-/-}$ mice. Similar to $C3^{-/-}$ mice, all grafts were rejected with a MST of 11 days after transplantation (Fig. 1a; $P = 0.3$), demonstrating that C3aR is necessary for DLI induced complement mediated tolerance.

C3aR on donor lymphocytes and recipient mice, but not donor skin grafts, is required for complement dependent transplant tolerance

Data so far described indicate that all three complement receptors tested may play a role in our model of HY specific tolerance, with experiments in $C3aR^{-/-}$ mice giving the most clear-cut results. Therefore, we concentrated subsequent investigations on C3aR. DLI preparations obtained from WT and $C3aR^{-/-}$ mice showed no difference in absolute or relative numbers of IA^{b+}, $CD11c^+$, $CD38^+B220^+IgM^+$, $CD3^+CD8^+$, $CD3^+CD4^+$, and $CD4^+CD25^+FoxP3^+$ cells (data not shown). To define which compartment is crucial for C3aR dependent tolerance, we performed skin transplants where only donor skin, DLI or recipient mice were C3aR deficient (Fig. 1b). Most of the WT mice receiving $C3aR^{-/-}$ donor skin had indefinite graft survival (88% >100 days after transplantation), comparable to WT donor skin ($P = 0.18$). In contrast, all $C3aR^{-/-}$ recipients and WT recipients of $C3aR^{-/-}$ DLI showed graft rejection with a MST of 10 and 9 days, respectively, at a tempo similar to C3aR deficiency in all three compartments ($P = 0.67$ and $P = 0.13$).

Absence of C3aR leads to an augmented donor specific $CD8^+$ T cell response

A recent study in C3 dependent tolerance has shown that complement deficient recipients have an increased donor specific $CD8^+$ cell response [9]. Therefore, we examined the donor specific $CD8^+$ T cell population in $C3aR^{-/-}$ and WT animals in the DLI induced transplant tolerance model, using the HY specific tetramer (Fig. 3). There was no difference in the number of total circulating $CD8^+$ T cells between the $C3aR^{-/-}$ and the WT group at day 9 (4% $CD8^+$/total lymphocytes vs. 10% $CD8^+$/total lymphocytes; $P = 0.47$) and 13 (16% $CD8^+$/total lymphocytes vs. 15% $CD8^+$/total lymphocytes; $P = 0.52$). However, $C3aR^{-/-}$ recipients had higher percentages of donor specific (HY $tetramer^+$) $CD8^+$ T cells compared to WT recipients at day 9 (1.4% HY $tetramer^+/CD8^+$ vs. 0.2% HY $tetramer^+/CD8^+$, $P = 0.008$) and 13 (4.3% HY $tetramer^+/CD8^+$ vs. 0.2% HY $tetramer^+/CD8^+$; $P = 0.002$) after transplantation. Thus, a lack of C3aR increases the magnitude of the donor specific $CD8^+$ T cell response following DLI in the HY mismatch transplant model.

Absence of C3aR leads to a decreased number of Treg with reduced suppressive function

In addition to an increased expansion of donor specific $CD8^+$ T cells, lower numbers of induced Treg may also be responsible for the inability of DLI to induce HY specific tolerance in $C3aR^{-/-}$ recipients. To test this hypothesis, we analyzed splenocytes harvested from WT and $C3aR^{-/-}$ animals following the DLI induced tolerance protocol using intracellular FoxP3 as a marker of Treg (Fig. 4a and b). Within the $CD4^+CD25^+$ cell population, there was a significantly higher percentage of $FoxP3^+$ cells in WT recipients at day 14 after transplantation (73%) compared to $C3aR^{-/-}$ recipients (62%; $P = 0.032$). Splenocytes from naïve WT and $C3aR^{-/-}$ female mice showed no difference in Treg cell numbers (data not shown).

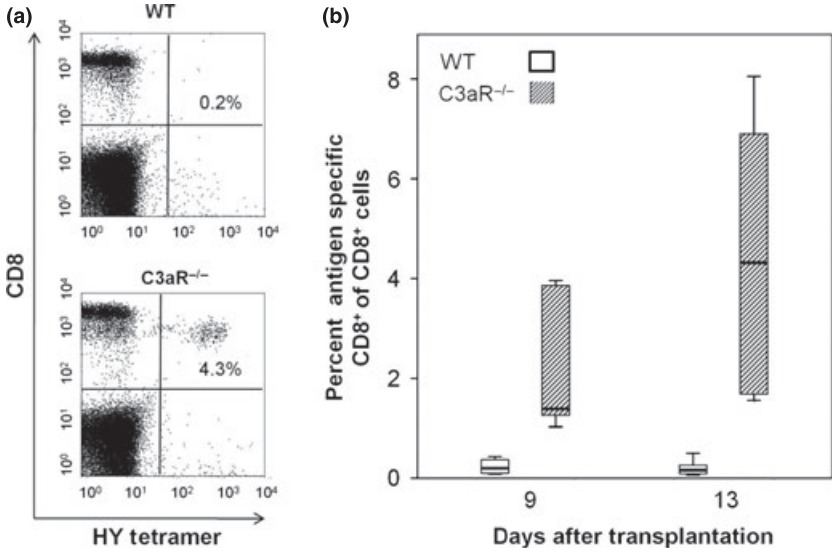


Figure 3 Analysis of donor antigen specific Teff. HY antigen-specific $CD8^+$ cells were detected using flow cytometric analysis at two different time points in peripheral blood of female recipients of a male skin graft and DLI. (a) Representative examples obtained from WT and $C3aR^{-/-}$ mice analyzed at day 13 after transplantation. Dot plots show HY antigen specific $CD8^+$ cells as percentage of PE conjugated HY tetramer positive cells (FL2) of total PerCP 5.5 conjugated rat anti-mouse CD8 positive cells (FL3). Percentages are median numbers of cells measured independently in six animals per group. (b) At day 9 and 13 after transplantation, there were more antigen-specific $CD8^+$ cells detected in $C3aR^{-/-}$ mice (hatched boxes) compared to WT mice (white boxes; 1.4% vs. 0.2%; $N = 5$; $P = 0.008$ and 4.3% vs. 0.2%; $N = 6$; $P = 0.002$). Error bars indicate a confidence interval of 95%.

A difference in the number of Treg between WT and $C3aR^{-/-}$ recipients after DLI tolerance induction may not necessarily be reflected in the overall Treg function. Therefore, we investigated Treg function of WT and $C3aR^{-/-}$ recipient splenocytes at day 14 after DLI tolerance induction *in vitro* using a CFSE-based suppression assay (Fig. 4c and d). There was no difference in $CD4^+CD25^+$ T cell suppression capability on $CD4^+CD25^-$ cells between WT and $C3aR^{-/-}$ recipients co-cultured at ratios of 1:1 (PI 1.54 vs. 1.46; $P = 0.41$) and 0.5:1 (PI 1.66 vs. 1.66; $P = 0.55$), respectively. However, at a ratio of 0.25:1, there is a trend toward a stronger $CD4^+CD25^-$ T cell suppression by WT recipient derived $CD4^+CD25^+$ T cells compared to $C3aR^{-/-}$ recipient derived cells (PI 1.78 vs. 1.95; $P = 0.056$) and at a ratio of 0.125:1, this difference becomes highly significant (PI 2.07 vs. 2.37; $P = 0.008$). Taken together, these data suggest that the absence of C3aR in the HY specific DLI induced tolerance model leads to a decreased number of Treg cells with a reduced suppressive function.

Absence of C3aR leads to imbalance of Teff and Treg at the primary site of immune response

To determine whether the imbalance between Teff and Treg in the periphery is also present at the local site of the immune response, we performed immunohistochemical staining of $C3aR^{-/-}$ and WT skin grafts. Applying the DLI induced tolerance protocol, a median of 57 $CD4^+$ and 18 $CD8^+$ lymphocytes per mm^2 were detected within leukocyte infiltrates in $C3aR^{-/-}$ skin grafts at day 7 post-transplantation (Fig. 5). There was no significant difference in Teff cell density compared to WT skin grafts ($P = 0.9$ for $CD4^+$ and for $CD8^+$ T-cells, respectively). In contrast, Treg cell numbers infiltrating skin grafts were lower in the absence of C3aR compared with WT controls ($6/mm^2$ vs. $31/mm^2$, $P = 0.029$). These data provide evidence for an imbalance in Teff and Treg lymphocytes in the skin grafts of $C3aR^{-/-}$ mice compared to WT mice.

Discussion

There is growing evidence for a contribution of the complement system in tolerance, including tolerance to allografts [8,9]. However, the exact mechanisms by which the complement system exerts its effect in transplant tolerance induction and maintenance are poorly understood. The objective of this study was to investigate the mechanisms involved in complement dependent tolerance. We found that the absence of complement receptors, especially C3aR, results in a failure of tolerance induction and Treg and Teff imbalance and that a dysfunction of DC induced Treg generation in the absence of complement could contribute to

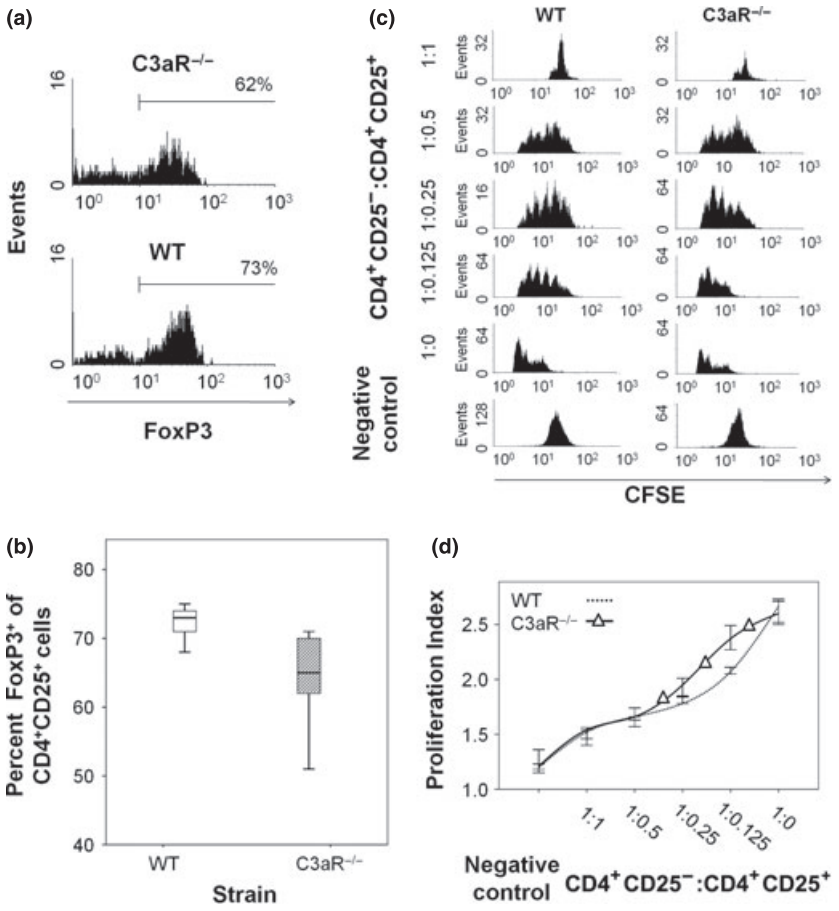
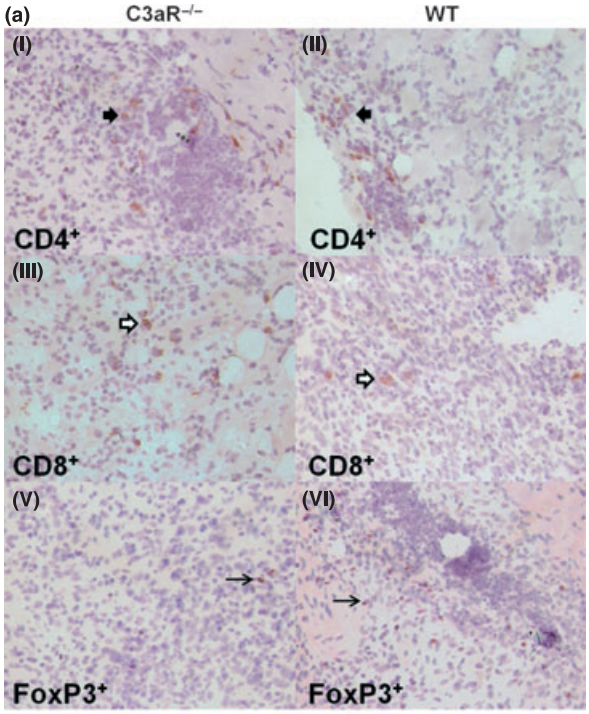


Figure 4 Analysis of Treg quantity and function. Numbers of Treg and their function were measured using flow cytometric analysis in spleens obtained from female recipients at day 14 after tolerance induction with DLI. (a) Representative examples obtained from WT and C3aR$^{-/-}$ mice. Median percentage of FoxP3 positivity (FL3) was calculated in histogram plots gated on FITC CD4 (FL1) and PE CD25 (FL2) double positive lymphocytes independently in five animals per group. (b) WT mice had a significantly higher percentage of FoxP3^{+} cells compared to C3aR$^{-/-}$ mice (73% vs. 62%; $N = 5$; $P = 0.032$). Error bars indicate a confidence interval of 95%. (c) Representative examples of *in vitro* CFSE stained CD4^{+}CD25^{+} proliferation analyzed in FL1 histogram plots. C3aR$^{-/-}$ Treg showed reduced suppressive function compared to WT Treg resulting in higher proliferation rates of C3aR$^{-/-}$ Teff. (d) A lower PI in WT recipients compared to C3aR$^{-/-}$ recipients at a CD4^{+}CD25^{+}:CD4^{+}CD25^{-} ratio of 1:0.25 and 1:0.125 reflects a higher suppressive function of WT Treg (1.78 vs. 1.95; $P = 0.056$ and 2.07 vs. 2.37; $P = 0.008$). Error bars indicate a confidence interval of 95%. Treg function was assessed independently in five mice per group.

this imbalance. The involvement of multiple complement receptors points toward the necessity of a network of complement regulatory pathways to establish tolerance induction rather than one crucial factor.

This is the first report, demonstrating that C3 produced by DC enhances Treg generation. It has been shown that IFN-gamma induced Treg generation involves conversion of non-Treg precursors [14] and that C3 split products produced by APC up regulate APC function [23,24]. Therefore, a paracrine effect of C3 on APC could indirectly lead to T cell conversion. Alternatively, C3 could act directly on T cells converting non-Treg precursors or expanding the existing Treg pool [23,24]. Another mechanism described for IFN-gamma induced Treg generation is cell death within the non-Treg population [14]. As C3 split products act as stimulating agents on T cells [23,24], it is unlikely that induction of cell death is the dominant mechanism of C3 enhanced Treg generation in our model here.

Our data suggest that DAF plays an important role for DLI induced tolerance. In the context of rejection, it has been proposed that DAF is acting directly via C3aR and complement component 5a receptor and indirectly on T cell function via APC, by limitation of C3a and complement component 5a generation affecting C3a/C3aR and complement component 5a/complement component 5a receptor interactions [19,20]. Alternatively, similar to its counterpart on human T cells, CD46, simultaneous engagement of DAF and T cell antigen receptor on mouse CD4^{+} T



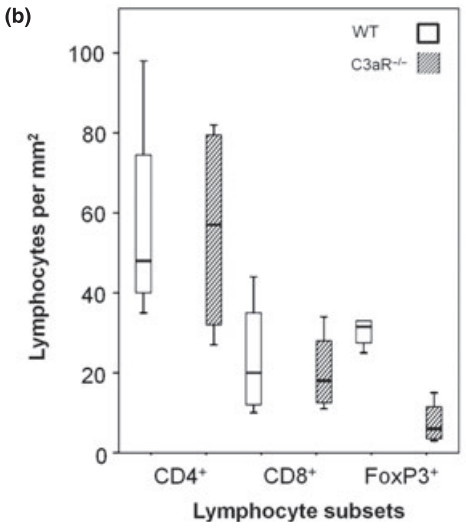


Figure 5 Immunohistologic analysis of Teff and Treg in skin grafts. Skin graft samples were analyzed at day 7 after tolerance induction with DLI. (a) Representative examples of lymphocyte subsets visualized with streptavidin horse radish peroxidase bound CD4, CD8, and FoxP3 (red brown) and Haematoxilin nuclear counter stain (purple). $CD4^+$ (I, II; black arrowhead), $CD8^+$ (III, IV; white arrowhead) and $FoxP3^+$ (V, VI; black arrow) cells are infiltrating skin grafts harvested from $C3aR^{-/-}$ (left column) and WT (right column) recipients. Original magnification ×400. (b) There was no difference in Teff density within skin graft infiltrates of WT ($N = 4$) compared to $C3aR^{-/-}$ mice ($N = 4$; 48 $CD4^+/mm^2$ vs. 57 $CD4^+/mm^2$; $P = 0.9$ and 20 $CD8^+/mm^2$ vs. 18 $CD8^+/mm^2$; $P = 0.9$ respectively), but significantly, more Treg could be counted in WT compared to $C3aR^{-/-}$ skin grafts (31 $FoxP3^+/mm^2$ vs. 6 $FoxP3^+/mm^2$; $P = 0.029$). Error bars indicate a confidence interval of 95%.

cells could results in a tolerogenic T cell phenotype, which contributes to DLI induced tolerance [25].

Complement receptor 3 contributes to DLI induced transplant tolerance in the same way as DAF according to our data presented. Previous studies have demonstrated that CR3 dependent tolerance is mediated through the binding of complement component iC3b to CR3 on APC [5,6]. As DLI induced tolerance is mediated through donor APC [26] it is possible that engagement of complement split product iC3b with its receptor CR3 on donor lymphocytes could be an important step in this model of transplant tolerance.

This is the first report that demonstrates C3aR as vital to the success of transplant tolerance induction. Data presented showed an absolute dependence on this receptor being present within the DLI and the recipient. It has been shown that HY specific DLI induced tolerance is dependent on HY antigen presentation by donor APC to recipient T cells [26] and APC from $C3aR^{-/-}$ mice have reduced antigen presenting function because of a lower surface expression of MHC class II, B7.1, B7.2, and CD40 [23]. A dysfunction to present HY antigen could explain why donor lymphocytes derived from $C3aR^{-/-}$ mice are not able to induce tolerance in our model. Activated T cells express C3aR [27], which is necessary for T cell modulation via C3a-C3aR engagement [21]. A lack of C3aR on recipient T cells could be responsible for the graft rejection in $C3aR^{-/-}$ recipients. These findings could explain why C3aR on DLI and on recipient cells are necessary for compliment mediated tolerance.

$C3aR^{-/-}$ recipients showed a consistent quantitative and qualitative imbalance between Treg and donor-specific Teff within the skin graft, the secondary lymphoid organs, and the peripheral blood. An increase in donor specific Teff function has also been described in a model of transplant tolerance in $C3^{-/-}$ and complement component $1q^{-/-}$ mice [9]. Recent study has demonstrated the impact of C3 and C3aR on direct and indirect T cell activation *in vitro* [22–24] suggesting that activation via C3aR could contribute to modulation of antigen-specific Teff in DLI induced tolerance. Treg have been implicated as important element in the development [28] and maintenance [29] of DLI induced tolerance in clinical studies. In addition, depletion of Treg has been shown to abrogate tolerance in an animal model of HY specific tolerance [30] and *in vitro* studies in human T cells suggests cell-surface detection of C3b or its metabolite iC3b could play a role in negative T cell regulation [25]. The expression of C3aR on Treg has not been described, therefore indirect action via APC on Treg, or direct on non-Treg precursors as discussed in the context of INF-gamma induced Treg generation and $C3aR^{-/-}$ donor APC, respectively, could be important for Treg development in WT mice. In addition to a quantitative

imbalance in Treg between WT and C3aR$^{-/-}$ recipients, we found a reduced suppressive function in Treg obtained from C3aR$^{-/-}$ mice. This functional deficiency could contribute to rejection in the absence of C3aR. However it is important to note that the study design cannot prove whether imbalance in Teff and Treg lymphocytes in C3aR$^{-/-}$ mice is causing the rejection process or is a result of ongoing rejection.

Taken together, our experiments suggest that multiple complement receptors play an important role in transplant tolerance induction. The absence of these components, especially C3aR, results in an ineffective tolerance induction that might be mediated through Treg and Teff imbalance. This provides new insights into the mechanisms of complement dependent tolerance induction and should be taken into account, especially in the development of complement modulating agents used in desensitization of solid organ transplant recipients and in treatment of graft rejection episodes.

Authorship

Gregor Bartel participated in the research design, in the performance of the research, and the data analysis and in writing the manuscript. Kathryn Brown, Richards Phillips, and Qi Peng participated in the performance of the research. Wuding Zhou, Steven H. Sacks, and Wilson Wong participated in the research design and in writing the manuscript.

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References

1. Kingsley CI, Nadig SN, Wood KJ. Transplantation tolerance: lessons from experimental rodent models. *Transpl Int* 2007: **20**: 828.
2. Orlando G, Hematti P, Stratta RJ, *et al.* Clinical operational tolerance after renal transplantation: current status and future challenges. *Ann Surg* 2010: **252**: 915.
3. Carroll MC. Complement and humoral immunity. *Vaccine* 2008: **26**(Suppl. 8): I28.
4. Kemper C, Atkinson JP. T-cell regulation: with complements from innate immunity. *Nat Rev Immunol* 2007: **7**: 9.
5. Sohn JH, Bora PS, Suk HJ, Molina H, Kaplan HJ, Bora NS. Tolerance is dependent on complement C3 fragment iC3b binding to antigen-presenting cells. *Nat Med* 2003: **9**: 206.
6. Hammerberg C, Katiyar SK, Carroll MC, Cooper KD. Activated complement component 3 (C3) is required for ultraviolet induction of immunosuppression and antigenic tolerance. *J Exp Med* 1998: **187**: 1133.
7. Schmidt J, Klempp C, Buchler MW, Marten A. Release of iC3b from apoptotic tumor cells induces tolerance by binding to immature dendritic cells in vitro and in vivo. *Cancer Immunol Immunother* 2006: **55**: 31.
8. Sacks S, Lee Q, Wong W, Zhou W. The role of complement in regulating the alloresponse. *Curr Opin Organ Transplant* 2009: **14**: 10.
9. Baruah P, Simpson E, Dumitriu IE, *et al.* Mice lacking C1q or C3 show accelerated rejection of minor H disparate skin grafts and resistance to induction of tolerance. *Eur J Immunol* 2010: **40**: 1758.
10. Stegall M, Tayyab D, Lynn C, Justin B, Patrick D, Gloor JM. Terminal complement inhibition decreases early acute humoral rejection in sensitized renal transplant recipients. *AJT* 2010; **10**: 39.
11. Patel H, Smith RA, Sacks SH, Zhou W. Therapeutic strategy with a membrane-localizing complement regulator to increase the number of usable donor organs after prolonged cold storage. *J Am Soc Nephrol* 2006: **17**: 1102.
12. Billingham RE, Medawar PB. Desensitization to skin homografts by injections of donor skin extracts. *Ann Surg* 1953: **137**: 444.
13. Yamaguchi Y, Tsumura H, Miwa M, Inaba K. Contrasting effects of TGF-beta 1 and TNF-alpha on the development of dendritic cells from progenitors in mouse bone marrow. *Stem Cells* 1997: **15**: 144.
14. Feng G, Gao W, Strom TB, *et al.* Exogenous IFN-gamma ex vivo shapes the alloreactive T-cell repertoire by inhibition of Th17 responses and generation of functional Foxp3+ regulatory T cells. *Eur J Immunol* 2008: **38**: 2512.
15. Brown K, Moxham V, Karegli J, Phillips R, Sacks SH, Wong W. Ultra-localization of Foxp3+ T cells within renal allografts shows infiltration of tubules mimicking rejection. *Am J Pathol* 2007: **171**: 1915.
16. Phillips RE, Sacks SH, Wong W. Critical role of C3 in transplant tolerance. *AJT* 2006: **6**: 892.
17. Zheng XX, Sanchez-Fueyo A, Sho M, Domenig C, Sayegh MH, Strom TB. Favorably tipping the balance between cytopathic and regulatory T cells to create transplantation tolerance. *Immunity* 2003: **19**: 503.
18. Heeger PS, Lalli PN, Lin F, *et al.* Decay-accelerating factor modulates induction of T cell immunity. *J Exp Med* 2005: **201**: 1523.
19. Pavlov V, Raedler H, Yuan S, *et al.* Donor deficiency of decay-accelerating factor accelerates murine T cell-mediated cardiac allograft rejection. *J Immunol* 2008: **181**: 4580.

20. Esposito A, Suedekum B, Liu J, *et al.* Decay accelerating factor is essential for successful corneal engraftment. *Am J Transplant* 2010: **10**: 527.
21. Strainic MG, Liu J, Huang D, *et al.* Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. *Immunity* 2008: **28**: 425.
22. Li K, Anderson KJ, Peng Q, *et al.* Cyclic AMP plays a critical role in C3a-receptor-mediated regulation of dendritic cells in antigen uptake and T-cell stimulation. *Blood* 2008: **112**: 5084.
23. Peng Q, Li K, Anderson K, *et al.* Local production and activation of complement up-regulates the allostimulatory function of dendritic cells through C3a–C3aR interaction. *Blood* 2008: **111**: 2452.
24. Peng Q, Li K, Patel H, Sacks SH, Zhou W. Dendritic cell synthesis of C3 is required for full T cell activation and development of a Th1 phenotype. *J Immunol* 2006: **176**: 3330.
25. Kemper C, Chan AC, Green JM, Brett KA, Murphy KM, Atkinson JP. Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature* 2003: **421**: 388.
26. Brennan DC, Mohanakumar T, Flye MW. Donor-specific transfusion and donor bone marrow infusion in renal transplantation tolerance: a review of efficacy and mechanisms. *Am J Kidney Dis* 1995: **26**: 701.
27. Werfel T, Kirchhoff K, Wittmann M, *et al.* Activated human T lymphocytes express a functional C3a receptor. *J Immunol* 2000: **165**: 6599.
28. Kishimoto K, Yuan X, Auchincloss H Jr, Sharpe AH, Mandelbrot DA, Sayegh MH. Mechanism of action of donor-specific transfusion in inducing tolerance: role of donor MHC molecules, donor co-stimulatory molecules, and indirect antigen presentation. *J Am Soc Nephrol* 2004: **15**: 2423.
29. Bushell A, Karim M, Kingsley CI, Wood KJ. Pretransplant blood transfusion without additional immunotherapy generates CD25+CD4+ regulatory T cells: a potential explanation for the blood-transfusion effect. *Transplantation* 2003: **76**: 449.
30. Benghiat FS, Graca L, Braun MY, *et al.* Critical influence of natural regulatory CD25+ T cells on the fate of allografts in the absence of immunosuppression. *Transplantation* 2005: **79**: 648.