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B cells assist allograft rejection in the deficiency of protein kinase c-theta

Wenwei Yan,[1] Rui Xu,[1] Lian Li Ma,[1] Wei Han,[2] Sunil K. Geevarghese,[1] Phillip E. Williams,[1] Roger Sciammas,[3] Anita S. Chong[3] and Deng Ping Yin[1]

1 Division of Hepatobiliary Surgery and Liver Transplantation, Department of Surgery, Vanderbilt University Medical Center, Nashville, TN, USA
2 Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA
3 Department of Surgery, University of Chicago School of Medicine, Chicago, IL, USA

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Correspondence
Deng Ping Yin MD, PhD, Departments of Surgery, Division of Hepatobiliary Surgery and Liver Transplantation, Vanderbilt University Medical Center, 1161 21st Avenue South, CC2308 MCN, Nashville, TN 37232, USA.
Tel.: 615 343 6990;
fax: 615 343 1355;
e-mail: dengping.yin@vanderbilt.edu

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Summary

We have previously shown that mice deficient in protein kinase C theta (PKCθ) have the ability to reject cardiac allografts, but are susceptible to tolerance induction. Here we tested role of B cells in assisting alloimmune responses in the absence of PKCθ. Mouse cardiac allograft transplantations were performed from Balb/c (H-2d) to PKCθ knockout ($PKC\theta^{-/-}$), PKCθ and B cell double-knockout (PBDK, H-2b) mice and wild-type (WT) C57BL/6 (H-2b) mice. PBDK mice spontaneously accepted the allografts with the inhibition of NF-κB activation in the donor cardiac allograft. Anti-B cell antibody (rituximab) significantly delayed allograft rejection in $PKC\theta^{-/-}$, but not in WT mice. Co-transfer of $PKC\theta^{-/-}$ T plus $PKC\theta^{-/-}$ B cells or primed sera triggered allograft rejection in $Rag1^{-/-}$ mice, and only major histocompatibility complex class II-enriched B cells, but not class I-enriched B cells, were able to promote rejection. This, together with the inability of $PKC\theta^{-/-}$ and $CD28^{-/-}$ double-deficient (PCDK) mice to acutely reject allografts, suggested that an effective cognate interaction between $PKC\theta^{-/-}$ T and B cells for acute rejection is CD28 molecule dependent. We conclude that T–B cell interactions synergize with $PKC\theta^{-/-}$ T cells to mediate acute allograft rejection.

Introduction

Protein kinase C theta (PKCθ) is expressed in lymphoid tissues and skeletal muscle, and high levels of expression are observed in $CD4^+$ and $CD8^+$ T cells, while B cells are completely devoid of PKCθ [1]. PKCθ has been reported to be essential for mature T cell activation [2], as well as for the development of thymocytes [3]. Activation of PKCθ leads to the initiation of downstream signaling pathways that activate the transcription factors nuclear factor κB NF-κB and AP-1 [4–7], while deficiency of PKCθ results in impaired receptor-induced stimulation of NF-κB and nuclear factor of activated T cells, causing defective T cell activation and reduced IL-2 production [8,9]. We have previously reported that $PKC\theta^{-/-}$ mice retain their ability to acutely reject cardiac allografts, as well as their susceptibility to tolerance induction in a murine cardiac transplantation model [10,11]. While WT T cells trigger allograft rejection, $PKC\theta^{-/-}$ T cells fail to elicit allograft rejection when adoptively transferred into T and B cell-deficient $Rag1^{-/-}$ mice [10]. These results indicate that $PKC\theta^{-/-}$ T cells have defective ability to be stimulated, and that B cells may be required to assist $PKC\theta^{-/-}$ T cell-mediated allograft rejection.

B cells constitutively expressing major histocompatibility complex (MHC) class I and class II present alloantigens to

T cells through direct and indirect recognition pathways. Upon activation, increased expressions of co-stimulatory signals B7-1 (CD80) and B7-2 (CD86) enable B cells to function as potent antigen-presenting cells (APCs) for alloreactive T cell activation [12]. Another consequence of the interaction of B cells with helper T cells is their terminal differentiation into plasma cells that secrete donor-specific antibodies (DSA) capable of mediating acute or chronic allograft rejection [13,14]. Despite these properties of B cells, the elimination of B cells using B cell knockout mice or anti-B cell antibody generally fails to prevent acute allograft rejection [15,16]. We hypothesize that B cells facilitate $PKC\theta^{-/-}$ T cell-mediated allograft rejection through: cognate interaction between B and T cells, DSA, activation of costimulatory signals, and inhibition of T_{reg} cells. Elimination or inhibition of B cells may induce graft acceptance in the deficiency of PKCθ. In this study, we will test this hypothesis using the mouse cardiac transplantation model.

Materials and methods

Mice and cardiac allograft transplantation

C57BL/6 (H-2b), BALB/c (H-2d), CD28 knockout ($CD28^{-/-}$, H-2b), B cell knockout (*mu*MT, H-2b), MHC class I knockout (H-2b), class II knockout (H-2b), and $Rag1^{-/-}$ mice were purchased from Jackson Laboratory. $PKC\theta^{-/-}$ (H-2b) mice were kindly provided by Dr. Zouming Sun, as described in previous reports [9,17]. *NF-κB-luc* mice (C57BL/6 and BALB/c), expressing luciferase under the control of a NF-κB promoter, were kindly provided by Dr. Timothy Blackwell, department of Medicine at Vanderbilt University Medical Center [18]. All experiments were performed according to protocols approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee. Mouse vascularized cardiac transplantation was performed as described in previous reports [19,20]. The rejection of cardiac allograft was defined as the day that the grafted heart completely stopped beating [21].

Bioluminescence imaging and tissue luciferase assay

Bioluminescence imaging (BLI) was performed at the different time points, post-transplantation, as previously described [11,22]. Briefly, after anesthesia with isoflurane, luciferin was injected intravenously at a dose of 50 mg/kg, and the mice were housed inside a light-tight box and imaged with an ICCD camera (Hamamatsu C2400-32, Hopkinton, MA). Luciferase density was expressed as regions of interest and represented NF-κB activation. Tissue NF-κB activation was determined by tissue luciferase assay using the Bradford Luciferase Reporter Assay kit (Promega, Madison, WI, USA) [22]. Luciferase activity was measured in the tissue by adding 100 μl of freshly reconstituted luciferase assay buffer to 20 μl of tissue lysate. Luciferase activity is expressed as relative light units (RLU) normalized for protein content.

Adoptive transfer of lymphocyte subsets and sera

The spleen was cut and gently minced, and cell suspensions were prepared. T and B cells were purified by a negative selection with mouse T and B cell isolation kits (R&D Systems Inc., Minneapolis, MN, USA), following manufacturer's instruction. The purity of lymphocyte subsets was analyzed by flow cytometry, and 90–95% of enriched T or B cell population was routinely obtained. These T cells (10×10^6) and B cells (10×10^6) were injected intravenously into $Rag1^{-/-}$ mice on the day of cardiac transplantation. Anti-donor antibodies, naïve and primed $PKC\theta^{-/-}$ sera were collected from naïve $PKC\theta^{-/-}$ mice and $PKC\theta^{-/-}$ mice engrafted with acute cardiac allografts on the day of rejection (7–10 days, post-transplantation).

Analyses of lymphocytes

Spleen cell suspensions or peripheral blood lymphocyte (PBLs) suspensions were obtained, and lymphocyte isolation solution (Cedarlane Labs, Burlington, NC, USA) was used to isolate mononuclear cells. The cells were fixed and permeabilized with Cytofix/Cytoperb. Cells were stained with phycoerythrin (PE)-, Fluorescein isothiocyanate (FITC)-, or APC-conjugated anti-CD4 (GK1.5), Foxp3 (FJK16) and B220 (RA3-6B2). B cells and $CD4^+$/$FoxP3^+$ subsets were analyzed using flow cytometry (LSRII, BD, San Jose, CA, USA).

Immunofluorescence examination

T- and B-cell zones in the spleen and T-cell and B-cell infiltration in the cardiac allografts were analyzed by immunofluorescence, as described previously [11]. Briefly, the spleens were harvested on days, 1, 4, and 7 and the cardiac allografts on day 4, post surgery, and embedded in Optimal Cutting Temperature media. The embedded tissues were sectioned and incubated with FITC-conjugated B cell (B220) and PE-conjugated T cell (2c11) antibodies (BD PharMingen, San Diego, CA, USA). The samples were mounted in slides with fluorescent mounting media (DakoCytomation, Inc., Carpinteria, CA, USA) and analyzed using a fluorescence microscope.

Statistics

The cardiac allograft survival days were presented as mean survival time ± standard deviation (MST ± SD). Statisti-

cal significance was analyzed by ANOVA (Statview 4.5, Abacus Concepts, Berkeley, CA, USA). For *in vitro* data analysis, results are presented as means ± standard error of mean (SEM) and comparisons between the values were performed using the two-tailed Student's *t*-test, and the level of significance was set at a $P < 0.05$.

Results

B cells are critical for the initiation of allograft rejection in the deficiency of PKCθ

In this study, H-2d cardiac allografts were engrafted into H-2b recipients, and the strain combinations are described in Table 1. To test whether acute rejection could be restored by additional B cells, purified $PKC\theta^{-/-}$ T cells (10×10^6) and B (10×10^6) were co-transferred into $Rag1^{-/-}$ mice on the day of transplantation. Figure 1a shows that $PKC\theta^{-/-}$ T cells alone failed to provoke cardiac allograft rejection, whereas all cardiac allografts were rejected by co-transfer of $PKC\theta^{-/-}$ T and B cells in $Rag1^{-/-}$ mice (MST ± SD = 11.2 ± 2.8 days). To further test the role of B cells in the facilitation of allograft rejection, PKCθ and B cell double-knockout (PBDK) mice were produced by cross-breeding $PKC\theta^{-/-}$ mice and *μMT* mice. Eighty percent (8/10) of PBDK mice transplanted with cardiac allografts (BALB/c, H-2d) without any immunosuppressant accepted their allografts (MST ≥ 100 days). In contrast, all allografts were acutely rejected in WT (MST ± SD = 8.2 ± 1.5 days) mice and *μMT* mice (MST ± SD = 8.5 ± 1.4 days, Fig. 1b). The PBDK mice, the majority of which accepted their allografts, showed a significant reduction in B cells in follicles of the spleen (Fig. 1c), but otherwise had normal development with neither discernible physical defects nor signs of autoimmune disease. While dominant T and B cell infiltrations in the cardiac allografts of WT recipients and reduced T cell infiltrations in the cardiac allografts of $PKC\theta^{-/-}$ and PCDK mice were observed, there was no B cell infiltration in the cardiac allograft of PBDK mouse at day 4, post surgery (Fig. 1d).

Table 1. Strain combinations of cardiac graft transplantation (in the orders of Figures 1, 2, and 5).

Donor	Recipient	Treatment	Animal#
Balb/c	$Rag1^{-/-}$	$PKC\theta^{-/-}$ T cells	6
Balb/c	$Rag1^{-/-}$	$PKC\theta^{-/-}$ T+B cells	5
Balb/c	$Rag1^{-/-}$	*PCDK* T+WT B cells	4
Balb/c	*WT*	No	10
Balb/c	$\mu MT^{-/-}$	No	4
Balb/c	$PKC\theta^{-/-}$	No	10
Balb/c	$PKC\theta^{-/-}/\mu MT^{-/-}$	No	10
Balb/c	*WT*	Anti-CD20	5
Balb/c	$PKC\theta^{-/-}$	Anti-CD20	9
Balb/c	$PKC\theta^{-/-}/CD28^{-/+}$	No	5
Balb/c	$PKC\theta^{-/-}/CD28^{-/-}$	No	8
Balb/c	$PKC\theta^{-/+}/CD28^{-/+}$	No	4
Balb/c	$CD28^{-/-}$	No	5
Balb/c	$Rag1^{-/-}$	$PKC\theta^{-/-}$ T+Class I^+ B cells*	4
Balb/c	$Rag1^{-/-}$	$PKC\theta^{-/-}$ T+Class II^+ B cells*	4
Balb/c	$Rag1^{-/-}$	$PKC\theta^{-/-}$ T+Primed sera*	5
Balb/c	$Rag1^{-/-}$	$PKC\theta^{-/-}$ T+Naïve sera*	4
Balb/c	$Rag1^{-/-}$	Primed sera*	5

*Class I^+ or Class II^+ B cells, MHC class I or class II-enriched B cells; Primed sera or DS sera, primed or donor-specific sera; Naïve sera, sera from naïve C57BL/6 mice.

Anti-B cell antibody significantly prolonged survival of allografts in $PKC\theta^{-/-}$ mice

To test whether elimination of B cells prevents allograft rejection in $PKC\theta^{-/-}$ recipients, anti-mouse CD20 mAb (rituximab, Genentech, Inc., S. San Francisco, CA, USA) was administered (0.1 mg per mouse × 2, i.p.) on the day of transplantation and at day 2, post surgery. Anti-CD20 mAb treatment significantly prolonged the survival of cardiac allografts, whereas the same regimen failed to prolong survival of cardiac allografts in WT mice. Six of nine cardiac allografts survived for more than 60 days in $PKC\theta^{-/-}$ mice treated with mAb (MST ± SD ≥ 45.6 ± 22.2 days, versus other groups, $P < 0.01$, Fig. 2a). Rituximab depleted more than 95% of peripheral B cells in mice over 1 month (Fig. 2b). We observed increased B cells in peripheral blood lymphocytes of rejected $PKC\theta^{-/-}$ mice by 1 month, post surgery, thus incomplete depletion or regeneration of B cells may have contributed to the observed allograft rejection (data not shown).

Alloimmune response in $PKC\theta^{-/-}$ mice is CD28 molecular dependent

CD28 signaling through PI3K results in recruitment of PKCθ to the cSMAC, activation of NF-κB, and induction of IL-2 transcription and allograft rejection. To define whether CD28 molecules are required for B cells to assist allograft rejection in the absence of PKCθ, PKCθ and CD28 double-knockout (PCDK) mice were produced. PCDK mice showed significant reduction in T cells in the areas surrounding small arterioles of the spleen, compared to WT, $PKC\theta^{-/-}$ and PBDK mice (Fig. 1c). All cardiac allografts were permanently accepted in homozygous PCDK ($PKC\theta^{-/-}/CD28^{-/-}$, MST > 100 days, $P < 0.001$), and rejected in heterozygous PCDK ($PKC\theta^{-/-}/CD28^{-/+}$, MST ± SD = 12.8 ± 3.2 days) and $PKC\theta^{-/+}/CD28^{-/-}$ mice (MST ± SD = 14.2 ± 2.8 days (Fig. 2c). Adoptive transfer of WT B cells (10×10^6) did not elicit allograft rejection in homozygous PCDK mice ($n = 4$, data not shown).

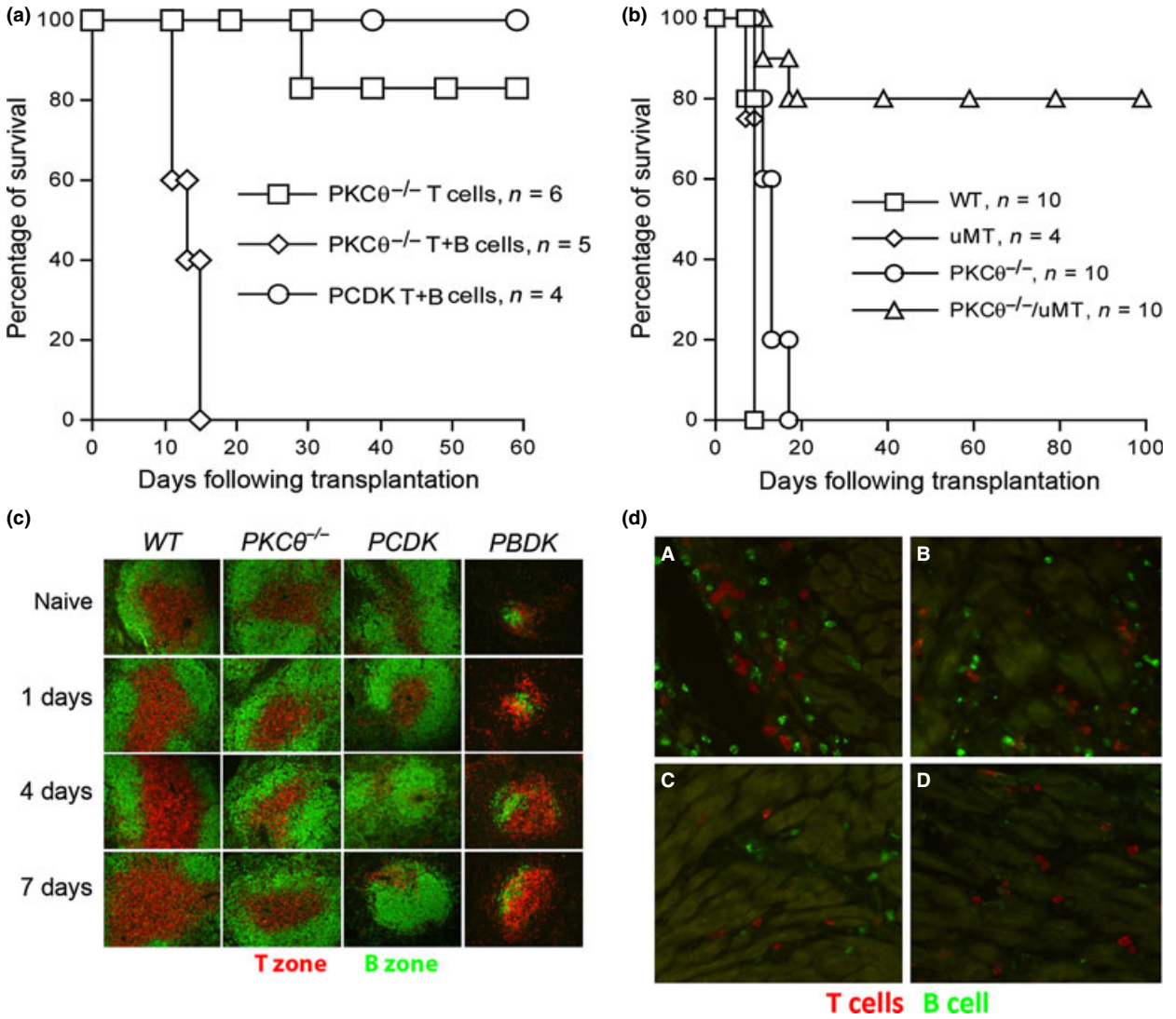

Figure 1 B cells assist cardiac allograft rejection in the absence of protein kinase C theta (PKCθ). (a) Adoptive transfer of *PKCθ*$^{-/-}$ T and B cells into *Rag1*$^{-/-}$ mice triggers cardiac allograft rejection. Co-transfer of *PKCθ*$^{-/-}$ or PCDK T cells (10×10^6) with wild-type (WT) B cells (10×10^6) into *Rag1*$^{-/-}$ mice on the day of cardiac allograft transplantation. (b) Cardiac allografts survived indefinitely in *PKCθ* and B cell double-knockout micec (PBDK). BALB/c (H-2d) cardiac allografts were transplanted into WT, *μMT*, *PKCθ*$^{-/-}$ and PBDK mice. PBDK mice vs. WT and *μMT* mice, $P < 0.01$. (c) Immunofluorescence examination of T and B zones in the spleen. T and B cell zones were examined by immunofluorescence before and after cardiac allograft transplantation. T cells in the periarteriolar lymphoid sheath were stained red, and B cells in the follicle were stained green and observed using a fluorescence microscope ($n = 3$ in each group). (d) Immunofluorescence examination of T and B cells in Balb/c cardiac grafts. T and B cell infilatrations in the cardiac allografts were examined by immunofluorescence at 4 days following transplantation. T cells were stained read and B cells were stained green. A. WT C57BL/6 mouse; B. *PKCθ*$^{-/-}$ mouse; C. PCDK mouse, and D. PBDK mouse ($n = 3$ in each group).

Elimination of B cells preserved regulatory T (T_{reg}) cell generation

We analyzed $CD4^+/FoxP3^+$ T cells in the spleen at 7 days, post-transplantation. The results in Fig. 3a show a significant reduction in the frequency of $CD4^+/FoxP3^+$ T cells in *PKCθ*$^{-/-}$ mice and a further reduction in PCDK mice. The percentage of $CD4^+/FoxP3^+$ T cells was comparable in *PKC*$^{-/-}$ and PBDK mice (PCDK, versus PKCθ$^{-/-}$ and PBDK, $P < 0.05$). Fig. 3b shows that the percentage of $CD4^+/FoxP3^+$ T cells modestly increased rituximab-treated *PKCθ*$^{-/-}$ mice engrafted with long-term surviving cardiac allografts (versus naïve *PKCθ*$^{-/-}$ or PKCθ-N, $P < 0.05$) and reduced in rejected *PKCθ*$^{-/-}$ mice (versus PKCθ-N, $P < 0.01$).

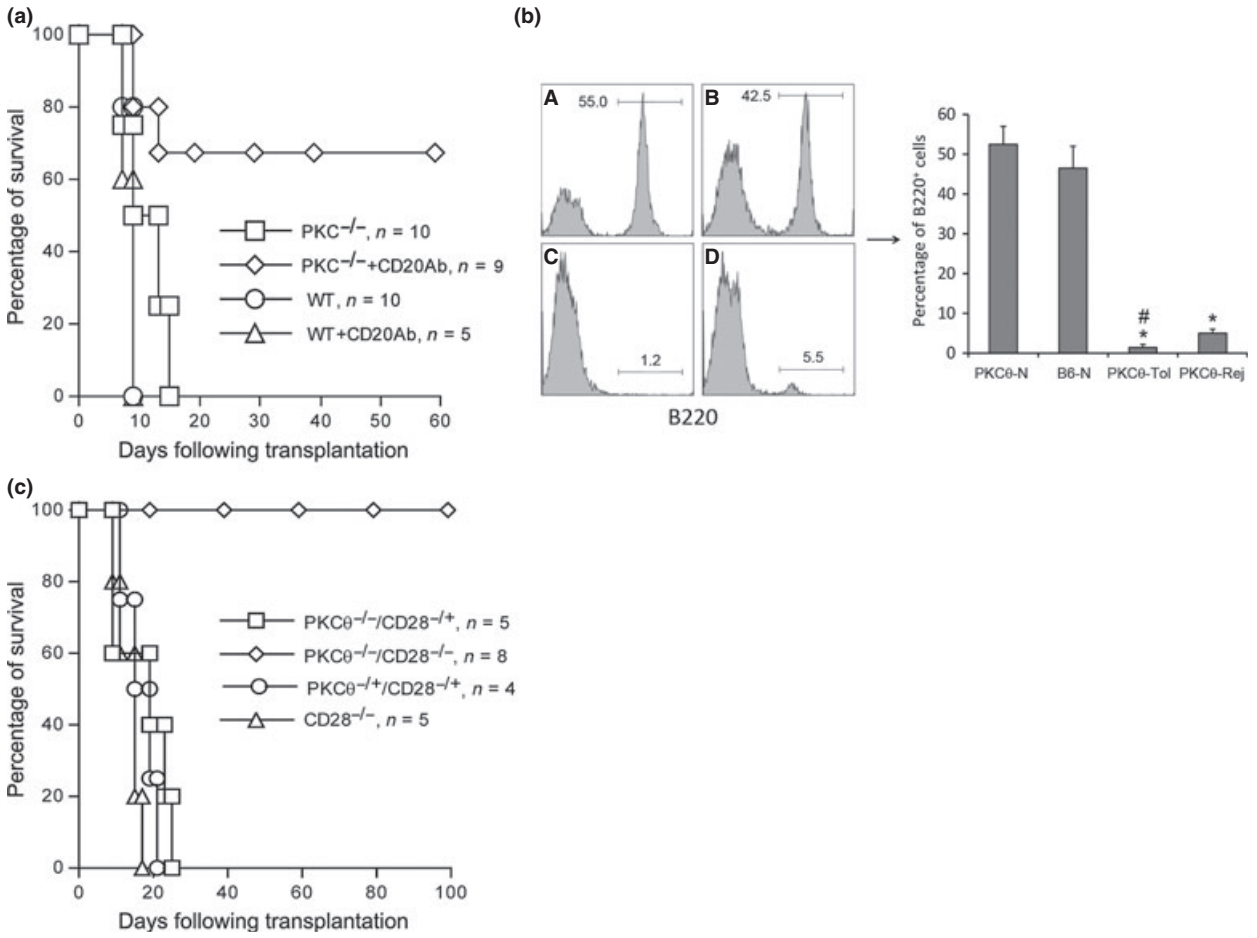

Figure 2 Inhibition of B cells or elimination of CD28 significantly prolongs survival of cardiac allografts in the absence of protein kinase C theta (*PKCθ*). (a) Inhibition of B cells significantly prolongs survival of cardiac allografts in *PKCθ*$^{-/-}$ mice. Anti-CD20 mAb (rituximab, 0.1 mg/mouse × 2, i.p.) prolonged survival of cardiac allograft in *PKCθ*$^{-/-}$ mice (versus all other groups, $P < 0.01$). (b) Acceptance of cardiac allografts in *PKCθ*$^{-/-}$ mice depends on effective depletion of B cells. Spleen cells were collected from *naïve PKCθ*$^{-/-}$ (A, PKCθ-N), *naïve* C57BL/6 (B, B6-N), rituximab-treated *PKCθ*$^{-/-}$ mice engrafted with long-term surviving cardiac allografts (C, PKCθ-Tol) and rituximab-*PKCθ*$^{-/-}$ mice with rejected cardiac allografts (D, PKCθ-Rej). *: vs. PKCθ-N, $P < 0.01$ and $^{\#}$: vs. PKCθ-Rej, $P < 0.05$. (c) Cardiac allografts survived indefinitely in PKCθ and CD28 double-knockout mice. BALB/c cardiac allografts (H-2d) were transplanted into homozygous and heterozygous PKCθ and CD28 knockout mice (H-2b).

Reduced NF-κB activation in the absence of PKCθ and B cells

Protein kinase C $\theta^{-/-}$ T cells have a functional defect in the activation of the NF-κB signaling pathway, which is imperative for the induction of alloimmune responses [23,24]. To investigate NF-κB activation in the cardiac allografts in the absence of PKCθ alone or in the absence of PKCθ and B cells, *NF-κB-luc* cardiac allografts (BALB/c, H-2d) were transplanted into WT, *PKCθ*$^{-/-}$ and PBDK mice (H-2b). The results in Fig. 4a show that luciferase activity was reduced in the cardiac allografts of *PKCθ*$^{-/-}$ and further reduced in PBDK mice, consistent with the rejection process inducing NF-κB activation in the cardiac allograft.

To further extend these findings, *PKCθ*$^{-/-}$ and PBDK mice expressing luciferase under control of the NF-κB promoter were used as recipients of cardiac allografts. The allografts were collected at day 7, post surgery, and tissue luciferase activity in the cardiac allografts was analyzed by a tissue luciferase assay. The results in Fig. 4b show reduced luciferase activity in *PKCθ*$^{-/-}$ mice consistent with our previous findings [22] and a further reduction in luciferase activity was observed in PBDK mice (*PKCθ*$^{-/-}$ vs. PBDK, $P < 0.05$).

MHC class II molecules in B cells elicit allograft rejection in the deficiency of PKCθ

B cells recognize and present alloantigens to MHC class II-restricted CD4$^+$ T cells. B cells (10×10^6) were isolated from class I and class II-deficient mice. These B cells were transferred with *PKCθ*$^{-/-}$ T cells (10×10^6) into *Rag1*$^{-/-}$

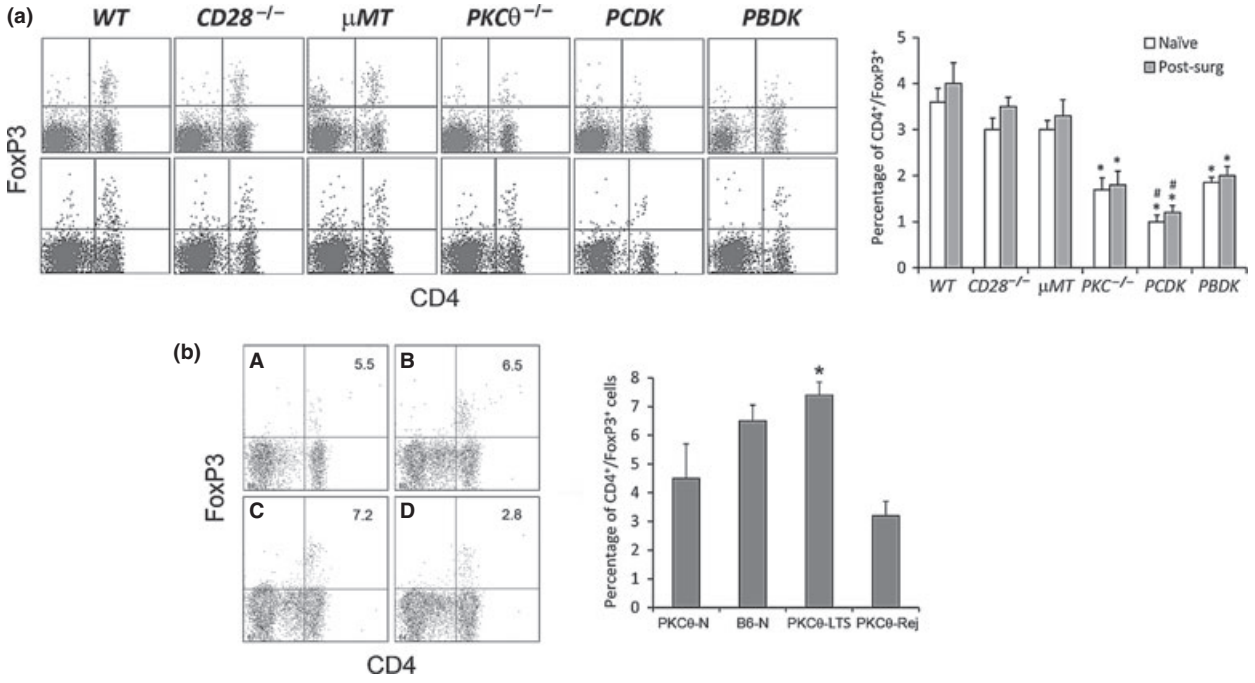

Figure 3 Flow cytometry analysis of *CD4*$^+$*/FoxP3*$^+$ T cells. (a) *CD4*$^+$*/FoxP3*$^+$ subsets in naïve spleen cells (upper panels) and in spleen cells of mice engrafted with cardiac allografts (lower panels). *: vs. wild-type (WT), $P < 0.01$, and $^{\#}$: *PCDK* vs. protein kinase C theta *PKCθ*$^{-/-}$ and *PBDK*, $P < 0.05$. (b) Inhibition of B cells with rituximab enhanced *CD4*$^+$*/FoxP3*$^+$ T cells in peripheral blood lymphocytes (*PBLs*). PBLs were collected from *naïve PKCθ*$^{-/-}$ (PKCθ-N); C57BL/6 (B6-N); *PKCθ*$^{-/-}$ mice with rituximab-induced long-term surviving cardiac allografts (PKCθ-LTS), and untreated *PKCθ*$^{-/-}$ mice with rejected cardiac allografts (PKCθ-Rej) at 7 days after transplantation. *: PKCθ-LTS, vs. PKCθ-N and PKCθ-Rej, $P < 0.01$. The results in Figure 3 are representative of more than three independent experiments.

mice. Class I-deficient (class II) B cells induced allograft rejection (MST ± SD = 13.0 ± 2.31 days), whereas in *Rag1*$^{-/-}$ mice transferred with class II-deficient (class I) B cells three of four cardiac allografts were accepted (Fig. 5a). These data suggest that interactions between Class II on B cells and *PKCθ*$^{-/-}$ CD4$^+$ T cells are necessary for the initiation of the acute rejection process.

Transfer of PKCθ$^{-/-}$ T cells and primed sera induced allograft rejection

To test whether DSA can promote *PKCθ*$^{-/-}$ T cell-mediated allograft rejection, primed sera were collected from *PKCθ*$^{-/-}$ mice at 7–10 days, post-transplantation when cardiac allografts (BALB/c, H-2d) were acutely rejected. Naïve *PKCθ*$^{-/-}$ T cells plus primed *PKCθ*$^{-/-}$ sera (0.2 ml) or naïve sera (0.2 ml) were injected (i.v.) into *Rag1*$^{-/-}$ mice on the day of cardiac allograft transplantation. While transfer of either naïve *PKCθ*$^{-/-}$ T cells or *PKCθ*$^{-/-}$ primed sera, alone, failed to induce allograft rejection, transfer of both naïve *PKCθ*$^{-/-}$ T cells and *PKCθ*$^{-/-}$ primed (but not naïve) sera induced allograft rejection (MST ± SD = 16.4 ± 5.4 days), suggesting that anti-donor serum is sufficient to synergize with *PKCθ*$^{-/-}$ T cells to induce acute allograft rejection (Fig. 5b).

Discussion

In this study, we investigated a critical role of B cells in facilitating *PKCθ*$^{-/-}$ T cell-mediated allograft rejection. While *PKCθ*$^{-/-}$ mice have the ability to elicit allograft rejection, adoptive transfer of *PKCθ*$^{-/-}$ T cells into T/B cell-deficient *Rag1*$^{-/-}$ mice fails to induce allograft rejection, as additional *PKCθ*$^{-/-}$ B cells are necessary for the induction of allograft rejection. Cardiac allografts are rejected in B cell-deficient *μMT* mice, but accepted by mice lacking both PKCθ and B cells (PBDK). Inhibition of B cells with anti-CD20 mAb significantly prolonged the survival of cardiac allografts in *PKCθ*$^{-/-}$ mice. Without CD28 molecules, *PKCθ*$^{-/-}$ T cells and B cells fail to elicit allograft rejection with reduced T cell infiltration in the allografts. The data collectively demonstrate that B cells assist T cells in eliciting cardiac allograft rejection independent of PKCθ; however, B cells cooperate with *PKCθ*$^{-/-}$ T cells to initiate allograft rejection in a CD28-dependent manner.

Upon activation of PKCθ, the downstream NF-κB signaling pathway is activated and mediates alloimmune

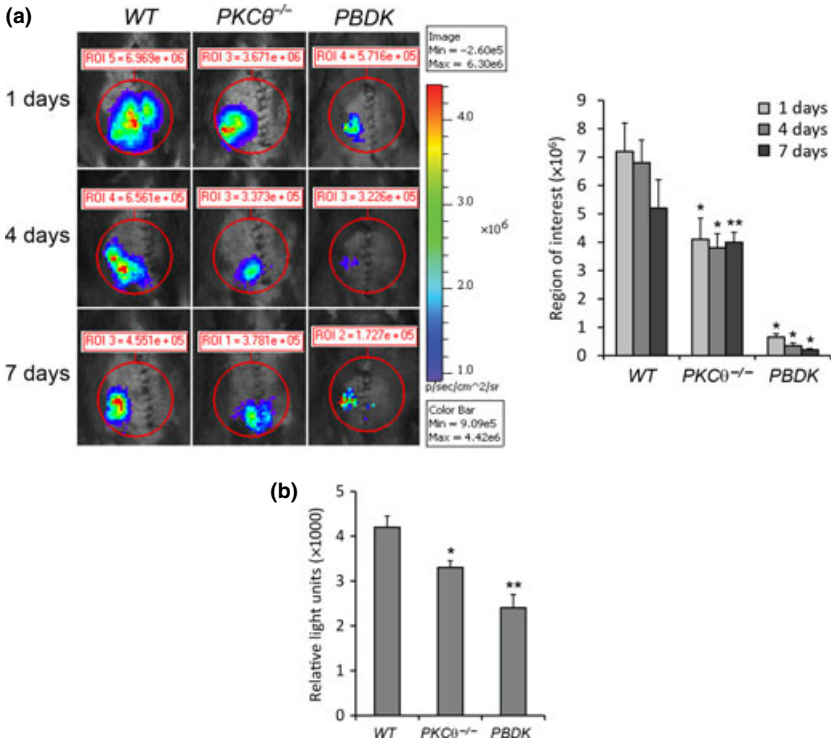

Figure 4 NF-κB activation and lymphocyte analyses in wild-type (WT_, protein kinase C theta $PKC\theta^{-/-}$ and PBDK mice. (a) Bioluminescence imaging (*BLI*) analysis of *NF-κB* activation. Luciferase transgenic mice under the control of the NF-κB promoter were used as donors of cardiac allografts. Luciferase, expressed as Regions of Interest (ROI), was measured by BLI at days 1, 4, and 7, post-transplantation. (b) Tissue luciferase analysis of *NF-κB* activation. Luciferase transgenic mice under the control of the NF-κB promoter were used as recipients of cardiac allografts. The cardiac allografts were collected and homogenized at 7 days after transplantation for tissue luciferase assays. *: vs. WT, $P < 0.01$ and **: vs. WT and $PKC\theta^{-/-}$, $P < 0.01$.

responses. NF-κB blockade enhances generation of T_{reg} cells through the regulation of direct and indirect antigen presentation [25]. Given the fact that NF-κB is one of the primary physiological targets of PKCθ, NF-κB activation will be diminished and the allograft is more susceptible to tolerance induction in the absence of PKCθ [10,11]. When NF-κB promoter-driven luciferase transgenic mice were used as donor, as well as recipient, reduced luciferase activity was observed in cardiac allografts of PKCθ-deficient mice. This suggests an inhibition of NF-κB activation [11]. The current results show that elimination of B cells further inhibits both donor- and recipient-derived NF-κB activation.

B cells can function as APCs and regulate the T cell responses that mediate acute allograft rejection [26]. Indeed our results show that co-transfer of $PKC\theta^{-/-}$ T cells with MHC class II-enriched B cells elicited allograft rejection in T/B cell-deficient $Rag1^{-/-}$ mice, whereas co-transfer of $PKC\theta^{-/-}$ T cells with MHC class I enriched B cells failed to do so. The results suggest that MHC class II, but not class I, plays a necessary role in B cell-assisted activation of $PKC\theta^{-/-}$ T cells, and that other APCs, such as dendritic cells, are not sufficient.

The cognate interaction between B cells and T cells leads to the differentiation of B cells into plasma cells secreting antibodies that mediate allograft rejection. In the absence of PKCθ, either B cells or alloantibodies have the ability to initiate allograft rejection. It has been reported by Burns *et al.* that alloantibodies can function as opsonins to facilitate antigen presentation and the activation of alloreactive T cells in a CD40–CD154-independent manner [27]. Indeed, we observed that naïve $PKC\theta^{-/-}$ T cells, in combination with donor-specific primed sera obtained from $PKC\theta^{-/-}$ mice bearing rejected cardiac allografts, but not naïve sera, elicited acute allograft rejection in $Rag1^{-/-}$ mice. $PKC\theta^{-/-}$ T cells and donor-specific sera, alone, failed to trigger allograft rejection. Our results suggest that primed $PKC\theta^{-/-}$ antibody can similarly assist $PKC\theta^{-/-}$ T cells to induce alloimmune responses. It must be noted that it is currently not clear whether the antibodies function as opsonins that then enhance APC function and the activation of the $PKC\theta^{-/-}$ T cells, or whether the antibodies function as effectors by binding to the graft, inducing inflammation in the graft in a complement-dependent manner, as described by Wasowska, *et al.* [28].

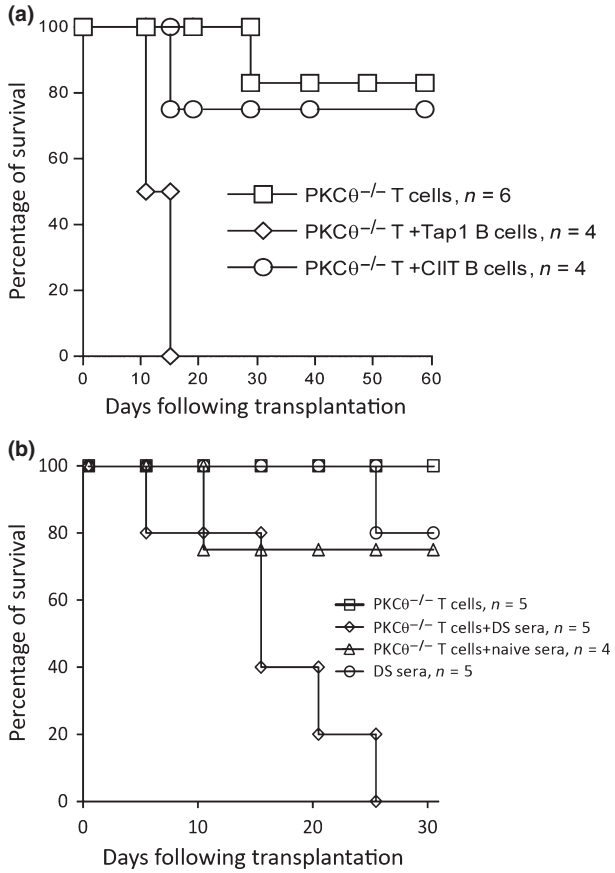

Figure 5 Adoptive transfer. (a) Major histocompatibility complexMHC class II mediates allograft rejection in the absence of protein kinase C theta (*PKCθ*). *PKCθ*$^{-/-}$ T cells (10×10^6) plus MHC class I-deficient B cells (PKCθ$^{-/-}$ T + Tap1 B cells, 10×10^6); *PKCθ*$^{-/-}$ T cells plus class II deficient B cells (PKCθ$^{-/-}$ T + CIIT B cells, 10×10^6) were transferred into *Rag1*$^{-/-}$ mice on the day of cardiac allograft transplantation. When PKCθ$^{-/-}$ T + Tap1 B cells group versus *PKCθ*$^{-/-}$ T cells group, MST ± SD ≥ 48.8 days, $P = 0.0166$, and PKCθ$^{-/-}$ T + CIIT B cells group versus *PKCθ*$^{-/-}$ T cells group, $P > 0.05$. (b) Transfer of *PKCθ*$^{-/-}$ T cells (10×10^6) plus primed sera (0.2 ml), but not *PKCθ*$^{-/-}$ T cells (10×10^6), *PKCθ*$^{-/-}$ T cells (10×10^6) plus naïve sera (0.2 ml) and primed sera alone (0.2 ml), induced allograft rejection in *Rag1*$^{-/-}$ mice. DS sera, donor-specific sera obtained from *PKCθ*$^{-/-}$ mice bearing rejected cardiac allografts at 7–10 days, post surgery. Naïve sera were collected from naïve *PKCθ*$^{-/-}$ mice.

Protein kinase C theta mediates thymocyte positive selection [29], and T cell population is reduced in the *PKCθ*$^{-/-}$ spleen and further abridged in the PCDK spleen. PKCθ signaling negatively regulates T_{reg} cell function [30], and peripheral T_{reg} cells are reduced in *PKCθ*$^{-/-}$ mice in a cardiac allograft transplantation model [11]. However, Dustin and his colleagues demonstrate that inhibition of PKCθ with compound 20 (C20; a potent and selective PKCθ inhibitor) enhances suppressive activity of human T_{reg} cells ($CD4^+/CD25^+$) [31]. WT and B cell-deficient mice had similar numbers of T_{reg} cells [28]. However, we show that while $CD4^+/FoxP3^+$ T cells are severely reduced in PCDK mice, $CD4^+/FoxP3^+$ T cells are preserved in PBDK mice, as compared to *PKCθ*$^{-/-}$ and PCDK mice. Furthermore, rituximab prolongs survival of cardiac allografts in *PKCθ*$^{-/-}$ mice with an increase in $CD4^+/FoxP3^+$ T cells. The mechanism for this is not clear, but it is possible that B cells block T_{reg} cell activity in the absence of PKCθ, and elimination of B cells enhances or promotes T_{reg} cell activation. Alternatively, it is likely owing to the loss of B cells, resulting in a compensatory increase in T_{reg} percentage, but not attributable to actual proliferation and selective expansion relative to total $CD4^+$ T cells.

In conclusion, this study provides insight into the mechanisms associated with the cognate interaction between T and B cells in assistance of the alloimmune response in the absence of PKCθ. *PKCθ*$^{-/-}$ T-mediated and B cell-assisted allograft rejection is dependent on CD28 molecules. Transfer of *PKCθ*$^{-/-}$ T and *PKCθ*$^{-/-}$ B cells or primed sera induces cardiac allograft rejection. Elimination or inhibition of B cells prolongs survival of cardiac allografts in the absence of PKCθ. Our findings also suggest that B cells assist *PKCθ*$^{-/-}$ T cells in initiating acute allograft rejection through MHC class II molecules. Thus, dual control of PKCθ in T cells and B cells provides a novel potential strategy for the prevention of allograft rejection.

Authorship

WY, RX and LLM: researched data. WH: analyzed luciferase activity. RS: analyzed B cell action. DPY: researched data and wrote manuscript. SKG, PEW and ASC: contributed by editing and discussing the manuscript.

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