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Apoptosis of Peripheral T Cells in Rodent Cardiac Allograft Recipients Induced by Donor-Specific Transfusion With Impaired Inducible Costimulator/B7 Homologous Protein Allorecognition

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ABSTRACT

Objective. To investigate apoptosis of the $CD8^+$ T cells (T_c) subpopulation in rodent cardiac allograft recipients, which were treated by donor specific transfusion combined with blockade of Inducible costimulator (ICOS)/B7 homologous protein (B7h) costimulation.

Methods. Donor hearts were heterotopically transplanted into the necks of recipient mice using Chen's technique. Postoperative graft survival was recorded. Both the percentage of $CD3^+CD8^+ICOS^+$ T_c in recipients' peripheral blood and the apoptosis of $CD8^+$ T_c in recipient draining lymph nodes were detected by flow cytometry analysis.

Results. In comparison with the allogeneic group, the survival of cardiac grafts was prolonged by combined treatment with 5×10^6 ICOS-Fc-targeted B cells on day 0 of transplantation and 10 mg/kg/d ICOS-Fc on days 0 to 6 (84.38 ± 29.14 days versus 7.00 ± 0.76 days, $P < .01$). The treatment group showed a stable $CD8^+T_c$ clone size in recipient peripheral blood ($49.4\% \pm 3.11\%$ versus $50.0\% \pm 2.46\%$, $P > .05$); however, the percentage of $CD3^+CD8^+ICOS^+$ T_c decreased significantly compared with the allogeneic group ($7.5\% \pm 2.02\%$ versus $14.0\% \pm 3.03\%$, $P < .05$). Compared with allogeneic group, apoptosis of the $CD8^+$ T_c subpopulation in recipient draining lymph nodes was upregulated significantly at postoperative 7 days in the treatment group ($19.53\% \pm 5.10\%$ versus $8.70 \pm 3.14\%$, $P < .05$).

Conclusion. Apoptosis of $CD8^+$ T_c in recipient draining lymph nodes was enhanced by pretreatment with donor specific transfusion and impaired ICOS/B7h allorecognition, which may have been associated with the variation in the $CD3^+CD8^+ICOS^+$ T_c subpopulation in peripheral blood and at least partially contributed to unresponsiveness toward cardiac allograft.

IMMUNOLOGIC BARRIERS remain the major challenqe in solid organ transplantation. Accumulated data from rodent models have indicated that long-term survival of allografts relied on both the induction and maintenance of unresponsiveness, which involves several mechanisms including apoptosis of alloreactive (allo-) T cells (T_c). Previous results have revealed transfer of cardiac allograft unresponsiveness after donor-specific transfusion (DST) combined with blockade of inducible costimulator (ICOS)/B7 homologous protein (B7h) allorecognition.[1] Using this

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model, we sought to investigate apoptosis among the $CD8^+$ T_c subpopulation in the early period after heterotopic cardiac transplantation across full major histocompatability complex mismatched strains using pretreatment with DST plus imparied ICOS/B7h allorecognition.

MATERIALS AND METHODS

Male mice of inbred strains BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) and aged 6 to 8 weeks were obtained from our Experimental Animal Center and maintained in pathogen-free conditions. Our Guidelines for the Care and Use of Laboratory Animals were followed in this study.

The recombinant murine ICOS-Fc, of which the extracellular domain of murine ICOS was fused to the Fc region of human immunoglobulin G1 via a polypeptide linker has been described previously (R&D Systems, Inc, USA).[2] EZ-Sep Mouse 1X was purchased from Dakewe Biotech (China). RPMI 1640, fetal bovine serum (FBS), phosphate-buffered saline (PBS), propidium iodide (PI), ammonium-chloride-potassium (ACK) Lysis Buffer and binding buffer were all purchased from Invitrogen (USA). PE-Cy5-CD3ε mAb, PE-CD8α mAb, CD16/CD32 mAb, Percp-CD3ε mAb, APC-CD8 mAb and Annexin V-FITC and PI Apoptosis Detection Kit were all purchased from BD Pharmingen (USA). FITC-ICOS mAb was purchased from eBioscience (Ireland, United Kingdom); B Cell Isolation Kit, from Miltenyi Biotec Inc (USA).

Resting splenocytes were harvested from mice by gradient centrifugation at 800*g* for 30 minutes using EZ-Sep Mouse 1× to remove red blood cells. Following washing twice in ice-cold RPMI1640 supplemented with 5% FBS, untouched splenic B cells were isolated by magnetic separation using the B Cell Isolation Kit, accordingto the manufacturers' protocols. ICOS-Fc-targeted donor B cells were prepared as follows[1,3]: splenic B cells harvested from BALB/c mice were incubated with ICOS-Fc at a concentration of 100 μg/mL at 4°C for 30 minutes in RPMI 1640 prior to extensive washing; finally, the cells were resuspended in PBS.

Donor hearts were heterotopically transplanted into the necks of recipient mice using Chen's technique.[4] Graft function was assessed daily by palpation; rejection was defined as the absence of detectable beat. Allografts that failed or graft recipients that died within 48 hours of surgery were considered to be technical failures and excluded from the analysis. Long-term allograft survival referred to survival time beyond 100 days; a median survival time $>$ 100 days was recognized as allograft acceptance.

Recipient mice were assigned to five groups: the isogeneic group including BALB/c donors and recipients did not undergo any treatment. In the allogeneic group, donor hearts from BALB/c mice were transplanted into C57BL/6 mice without treatment. A combination of 5×10^6 ICOS-Fc-targeted B cells on day 0 and 10 mg/kg/d ICOS-Fc on days 0 to 6[1] were given to a treatment of donor BALB/c mice grafts into recipient C57BL/6 mice. Similarly, ICOS-Fc was administered at a dose of 10 mg/kg/d for 7 days to the ICOS-Fc group. The DST group received 5×10^6 isolated donor B cells on the day of transplantation. Each injection was given in a total vollume of 200 μL PBS via the tail vein of the recipient.

Analysis of the T_c subpopulation in periperal blood was performed 7 days postoperatively. Briefly, periperal blood collected from recipient mice and treated with 50 U/mL heparin sodium, underwent red blood cell (RBC) lysis using ACK buffer. RBC-depleted splenotcytes were washed in PBS containing 5% FBS. Unlabeled anti-CD16/CD32 was used to block FcR binding; 1×10^6 cells were stained with PE-Cy5-CD3ε mAb, PE-CD8α mAb, or FITC-ICOS mAb for flow cytometry analysis on a EPICS XL (Beckman-Coulter), to detect the percentage of $CD3^+CD8^+$ $ICOS^+$ T_c.

The draining bilateral axillary and lateral axillary lymph nodes harvested at 7 days after heart transplantation yielded single-cell suspensions that were washed in PBS containing 5% FBS. Unlabeled anti-CD16/CD32 was used to block FcR binding. Then 1×10^6 cells stained with Percp-CD3εmAb and APC-CD8 mAb were resuspended in apoptosis binding buffer according to the manufacturer's instructions. After incubation with Annexin V-FITC for 15 minutes the cells were stained with PI prior to detection. Using a gate on $CD3^+CD8^+$ lymphocytes, the percentage of Annexin V^+PI^- cells was determined by four-color flow cytometry with analysis employing CellQuest acquisition software.

Survival data were analyzed using the Kaplan-Meier method with the log-rank test to evaluate the significance of survival differences between groups. Student *t* test was used to compare percentages of $CD8^+$ T_c subpopulation apoptosis with $P < .05$ considered to be significant statistically.

RESULTS

Survival of Grafts

Table 1 shows all of the isogeneic cardiac grafts to have survived more than 100 days. However, allografts were rejected within 8 days postoperatively among the allogeneic group (7.00 ± 0.76 days, $n = 8$). In comparison, allograft survival was prolonged by either ICOS-Fc (13.13 ± 1.55 days, $n = 8$, $P < .05$) or 5×10^6 isolated donor B cells (21.50 ± 2.56 days, $n = 8$, $P < .05$). Furthermore, graft survival in combined DST and ICOS/B7h blockade treatment group was significantly prolonged compared with the allogeneic group: 84.38 ± 29.14 days ($n = 8$; $P < .01$).

Variation of $CD8^+$ T_c Subpopulation in Peripheral Blood of Recipient Mice

As shownin Fig 1, the percentage of total $CD8^+$ T_c in the peripheral blood of allogeneic recipients increased markedly on day 7 after surgery, compared with that in the isogeneic group (50.0% ± 2.46% versus 31.7% ± 2.33%, $P < .05$). Similar results were observed in the treatment group (49.4% ± 3.11% versus 50.0% ± 2.46%, $P > .05$). Consistent with that observation, the percentage of $CD3^+CD8^+ICOS^+$ T_c subpopulation in the allogeneic group were much higher than that in the isogeneic group (14.0% ± 3.03% versus 1.6 ± 0.76%, $P < .005$); however, the combined treatment group showed a significantly decreased percentage of $CD3^+CD8^+ICOS^+$ T_c compared

Table 1. Prolonged Allograft Survival Achieved by the Combined Treatment of DST and ICOS/B7h Blockade

Group	*n*	Graft Survival (d)	*P* Value (compared with allogeneic group)
Isogenic group	8	>100	
Allogeneic group	8	7.00 ± 0.76	
DST group	8	13.13 ± 1.55	<.05
ICOS-Fc group	8	21.50 ± 2.56	<.05
Treatment group	8	84.38 ± 29.14	<.01

DST, donor-specific transfusion; ICOS, inducible costimulator; B7h, B7 homologous protein.