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Impact of Regulatory T Cells on Innate Immune Cells in a Pre-Sensitized Heart Transplant Model

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Although our previous studies revealed the role of Tregs (regulatory T cells) and MDSCs (myeloid-derived suppressor cells) in a pre-sensitized cardiac transplant model, interplay between Tregs and NK cells, neutrophils, and macrophages remain undefined.

Material/Methods: Mice heart transplantation with skin pre-sensitization was performed, in which prolonged-cold ischemia time (PCI) was used for donor treatment. Syngeneic heterotopic heart transplant recipients with PCI were treated with PC61 (monoclonal anti-CD25 antibodies), adoptive cell transfer with Tregs, and rapamycin.

Results: We unveiled that both rapamycin treatment and adoptive transfer of Tregs could lead to a remarkable decrease of frequency of splenic Gr1+ cells (P=0.058 and P=0.016, respectively). Although administration of PC61 did not affect frequency of splenic Gr1+ cells, it dramatically increased frequency of splenic F4/80+ macrophages (P=0.052). Intriguingly, use of both exogenous PC61 and rapamycin induced a dramatic augmentation of frequency of Gr-1+ neutrophils in the grafts (PC61: P=0.00029; rapamycin: P=0.0096). Noticeably, all different regimens including PC61, rapamycin, and adoptive transfer of Tregs, consistently resulted in a remarked augmentation of frequency of F4/80+ macrophages within grafts (PC61, P=0.0013; rapamycin, P=0.015; Tregs transfer, P=0.013). Although rapamycin and adoptive transfer of Tregs did not affect frequency of NK1.1+ cells, administration of PC61 dramatically increased frequency of NK1.1+ cells within grafts (P=0.033).

Conclusions: Tregs depletion or Tregs induced by rapamycin or exogenous cell transfer could affect frequencies of both splenic and intragraft neutrophils, macrophages, and NK cells, but not splenic NK cells. Our data might shed light on understanding sensitized transplant biology.

MeSH Keywords: Cold Ischemia • Heart Transplantation • Skin Transplantation • T-Lymphocytes, Regulatory

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Background

B and T cells are routinely highlighted to reduce allogeneic sensitization in solid organ transplantation [1]. Innate immune cells such as natural killer (NK) cells, macrophages, Gr1+ neutrophils, and myeloid-derived suppressor cells (MDSCs) are not likely to be underscored. Their roles remain unclear in the process of sensitized transplantation. Our past studies revealed that MDSCs played an important role in mouse pre-sensitized transplantation. Depletion of regulatory T cells (Tregs) could increase both peripheral and intragraft CD11b+Gr1-low frequency [2]. Endogenous Tregs promoted by rapamycin or adoptive transfer of exogenous wild-type Tregs could prevent the infiltration of Gr-1+ neutrophils and T lymphocytes [3]. Utilization of IL-6 deficient heart grafts caused a significant decrease of CD4-CD8-NK1.1+ and CD11b+Gr1^{high} cells frequency and a dramatic increase of CD11b+Gr1^{low} frequency in the recipient's spleen [4].

Although our previous studies revealed the interactive role of inflammatory cytokine IL-6, Tregs, and MDSCs in a pre-sensitized cardiac transplant model [2,3], interplay between Tregs and NK cells, neutrophils, and macrophages were not well-defined. Therefore, we further performed experimental transplantation to investigate it. It was identified that Tregs depletion or Tregs induced by rapamycin or exogenous cell transfer could affect frequencies of both splenic and intragraft neutrophils, macrophages, and NK cells, but not splenic NK cells.

Material and Methods

Mice

Inbred male mice 8- to 12-weeks old, including C57BL/6 (B6; H-2^b) and BALB/c (B/c; H-2^d) obtained from the Jackson Laboratories (Bar Harbor, ME) were used in the transplantation experiments. All mice were housed under pathogen-free facilities at the Center for Life Science (Boston, MA, USA). Experimental studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Beth Israel Deaconess Medical Center.

Sensitized transplant model with skin/abdominal cardiac transplantation

Tail skin patches from Balb/c donors (~0.5 cm in diameter) were transplanted onto the flank of C57BL/6 recipients. Seven days later, these pre-sensitized hosts underwent heterotopic abdominal cardiac transplantation by using wild-type C57BL/6 or Balb/c donors as described previously [5]. Briefly, mice were anesthetized through intraperitoneally injecting 20 mg/kg of xylazine and 100 mg/kg of detamine. After grafts were perfused with cold Custodiol HTK (histidine-tryptophan-ketoglutarate) solution (Bensheim, Germany), they were then stored at 4°C for 30 to 40 minutes or 5.5 to 8 hours. The donor aorta was sutured to the recipient's abdominal aorta by end-to-side anastomosis. The donor pulmonary artery was stitched with the recipient's inferior vena cava by end-to-side anastomosis. The graft function was daily observed by detecting donor heart-beat palpation. Donor hearts that did not beat within 1-day post-transplantation were excluded (Figure 1).

Experimental groups are shown in Table 1. All wild-type C57BL/6 hosts among 4 groups pre-sensitized by Balb/c skins at transplant day -7 were transplanted with C57BL/6 hearts plus comparable prolonged cold ischemia (PCI). Group 1 with PCI (6.9±1.0 hours) did not receive any treatment as negative controls (G1). Group 2 with PCI (6.8±0.7 hours) received monoclonal anti-CD25 antibodies (PC61). Group 3 with PCI (6.5±0.8 hours) received rapamycin treatment. Group 4 with PCI (6.8±1.5 hours) received adoptive transfer of Tregs. All transplanted cardiac grafts were harvested on day 3 post-transplantation to analyze splenic and intragraft immune status.

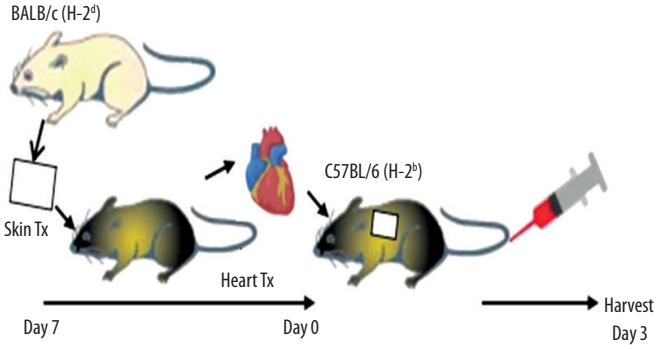

Figure 1. Diagrammatic sketch of experimental design; tx, transplantation.

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Table 1. Characteristics of experimental groups.

Group	n	Skin for sensitization	Donor for HTx	Tregs (0.5×10^6)	PC61	PCI (hrs)	Rapamycin (3 mg/kg BW)
G1	4	BALB/c	C57BL/6	No	No	6.9±1.0	No
G2	5	BALB/c	C57BL/6	No	Yes	6.8±0.7	No
G3	5	BALB/c	C57BL/6	No	No	6.5±0.8	Yes
G4	4	BALB/c	C57BL/6	Yes	No	6.8±1.5	No

Rapamycin treatment

Rapamycin at 1.0 mg/mL was initially suspended in 0.2% carboxymethylcellulose (Sigma) and then dispersed after ultrasonic disruption for 5 minutes as described previously [5]. A subclinical, non-tolerizing dose (3 mg/kg, Henry Schein, Melville, NY, USA) of rapamycin was intraperitoneally administered on transplant days –3 to 3 until graft harvest.

Tregs depletion *in vivo*

Tregs depletion was achieved by intraperitoneal injection of 0.5 mg of monoclonal anti-CD25 antibodies (Clone PC61, Bio Express, West Lebanon, NH, USA) on transplant day –1, followed by 0.25 mg on day 1 after heart transplantation. Analysis of CD4+CD25+ cells in the peripheral blood was made to confirm Tregs depletion on day 3 post-transplant via flow cytometry.

Cell sorting and adoptive cell transfer of Foxp3+ Tregs

The MoFlo high-speed cell sorter (DakoCytomation, Ft. Collins, CO, USA) was routinely utilized for cell sorting. Foxp3+ Tregs were collected from spleen and the lymph nodes from Foxp3-GFP reporter mice. Afterwards, single-cell suspension was achieved in complete RPMI-1640 medium. The enriched cells were then labeled with PE-anti-CD44 and FITC-anti-CD4 (eBioscience, San Diego, CA, USA). Thereafter, CD4+CD44+Foxp3-GFP+ cells population was gated and sorted. The purity of those sorted cells was consistently higher than 96%, as we previously reported [2]. The sorted Foxp3-GFP+ Tregs were adoptively transferred into the transplant hosts via their tail veins (0.5×10^6 cells/host).

Isolation of graft-infiltrating leukocytes (GILs)

The transplanted grafts were consistently harvested on day 3 after cardiac transplantation. The grafts were minced and then incubated in RPMI-1640 medium containing 0.5 mg/mL collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 37°C. After twice washing, viable mononuclear cells were carefully isolated by Ficoll density gradient centrifugation. Tissue debris was routinely removed by 70-µm cell-strainer. Afterwards, the isolated cells at the interface were recovered and washed thrice with RPMI 1640 medium supplemented with 10% FBS.

Antibodies and reagents

Immunofluorescence analysis and mAbs

The isolated cells were directly labeled with fluorescently conjugated monoclonal antibodies (mAbs). All mAbs used for cell surface staining were obtained from eBioscience or BD Pharmingen (San Diego, CA, USA). For FACS staining, anti-mouse CD3 (Clone 145-2C11) PE-Cy 7-conjugated mAb, anti-mouse anti-mouse CD8 (Clone 53–6.7) PE-conjugated mAb, CD4 (Clone H129.19) FITC-conjugated mAb, and Alexa Fluor® 700-conjugated anti-mouse Gr-1/Ly-6G (Clone RB6-8C5) mAb were used to label live T cells. All samples were subject to an LSRII (BD Biosciences, Mountain View, CA, USA). Data was then analyzed by using FlowJo 7.5 software (Tree Star, Ashland, OR, USA), as we reported previously [2].

Statistical analysis

Acquired data were analyzed through an unpaired Student *t*-test by using the statistical software SPSS (SPSS Inc., Chicago, IL, USA). Statistical significance was considered when $P<0.05$. The FACS data were expressed as mean ±SD.

Results

The frequency of Gr1+ neutrophils, F4/80+ macrophage, NK1.1+ cells in the spleen of the transplant recipients

Treatment with rapamycin resulted in a significant decrease of frequency of splenic Gr1+ cells ($P=0.058$), whereas adoptive cell transfer of Tregs led to an extremely decrease of frequency of splenic Gr1+ cells ($P=0.016$). Although administration of PC61 did not affect the frequency of splenic Gr1+ cells, it remarkably increased frequency of splenic F4/80+ macrophages ($P=0.052$). However, frequency of splenic NK1.1+ cells was not influenced irrespective of any treatment of PC61, adoptive transfer of Tregs or rapamycin ($P>0.05$) (Figure 2).

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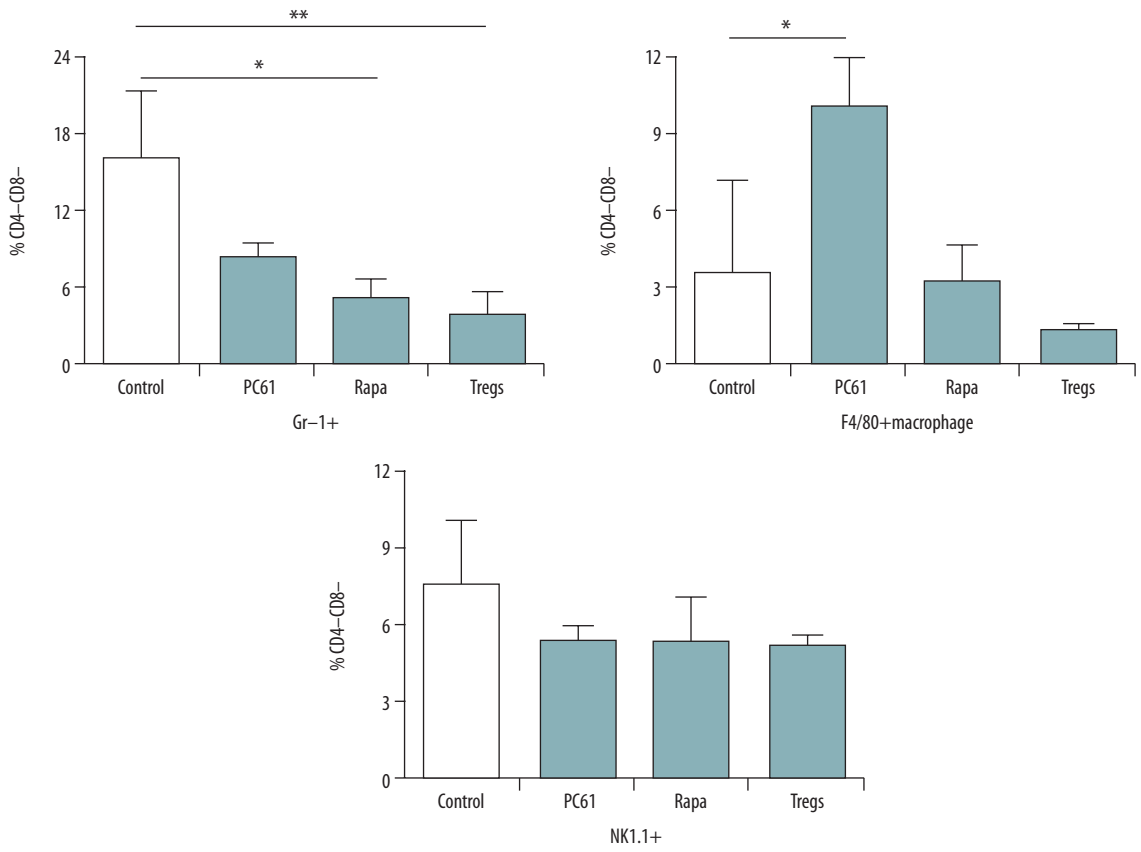

Figure 2. The frequency of Gr1+ neutrophils, F4/80+ macrophage, NK1.1+ cells in the spleen of the transplant recipients. These splenic cells were collected for fluorochrome-labeling and subject to FACS analysis on day 3 after transplant. Statistical analysis was performed for the groups G1 (Cont), G2 (PC61), G3 (Rap), and G4 (Tregs).

The frequency of Gr1+neutrophils, F4/80+ macrophage, NK1.1+ cells within cardiac graft of the transplant recipients

Intriguingly, different experimental findings were achieved within transplanted grafts. Use of both exogenous PC61 and rapamycin induced a dramatic augmentation of frequency of Gr-1+ neutrophils in the grafts (PC61: P=0.00029; rapamycin: P=0.0096). However, adoptive transfer of Tregs did not induce any influence on Gr1+ frequency within grafts (P>0.05). Noticeably, all different regimens including PC61, rapamycin, and adoptive transfer of Tregs consistently resulted in a remarked augmentation of frequency of F4/80+ macrophages within grafts (PC61, P=0.0013; rapamycin, P=0.015; Tregs transfer, P=0.013). Although rapamycin and adoptive transfer of Tregs did not affect frequency of NK1.1+ cells, administration of PC61 dramatically increased frequency of NK1.1+ cells within grafts (P=0.033) (Figure 3).

Discussion

In our present study, we utilized the pre-sensitized PCI-mediated transplant model based upon the following two reasons. First, empirical transplant recipients frequently undergo blood transfusion, pregnancies, failed transplant, and other events, which lead to alloantigen-sensitization conditions [6]. Second, scarcity of donor organs coerces utilization of remote donors, which leads to a prolongation of cold-ischemic time. The PCI time then induces graft injuries and an increase of morbidity of cardiac transplantation [7]. Therefore, it is required to investigate interplay between regulatory T cells and NK cells, neutrophils, and macrophages in a pre-sensitized PCI-mediated transplant model.

Cold ischemic times of approximately 7 hours were chosen in our model, as we observed it might cause a significant impact on allogeneic cardiac graft such as graft inflammatory injury [3]. In our present study model, pro-inflammatory events including cytokines storm would interact with pre-sensitized immune responses. Furthermore, our previous study revealed

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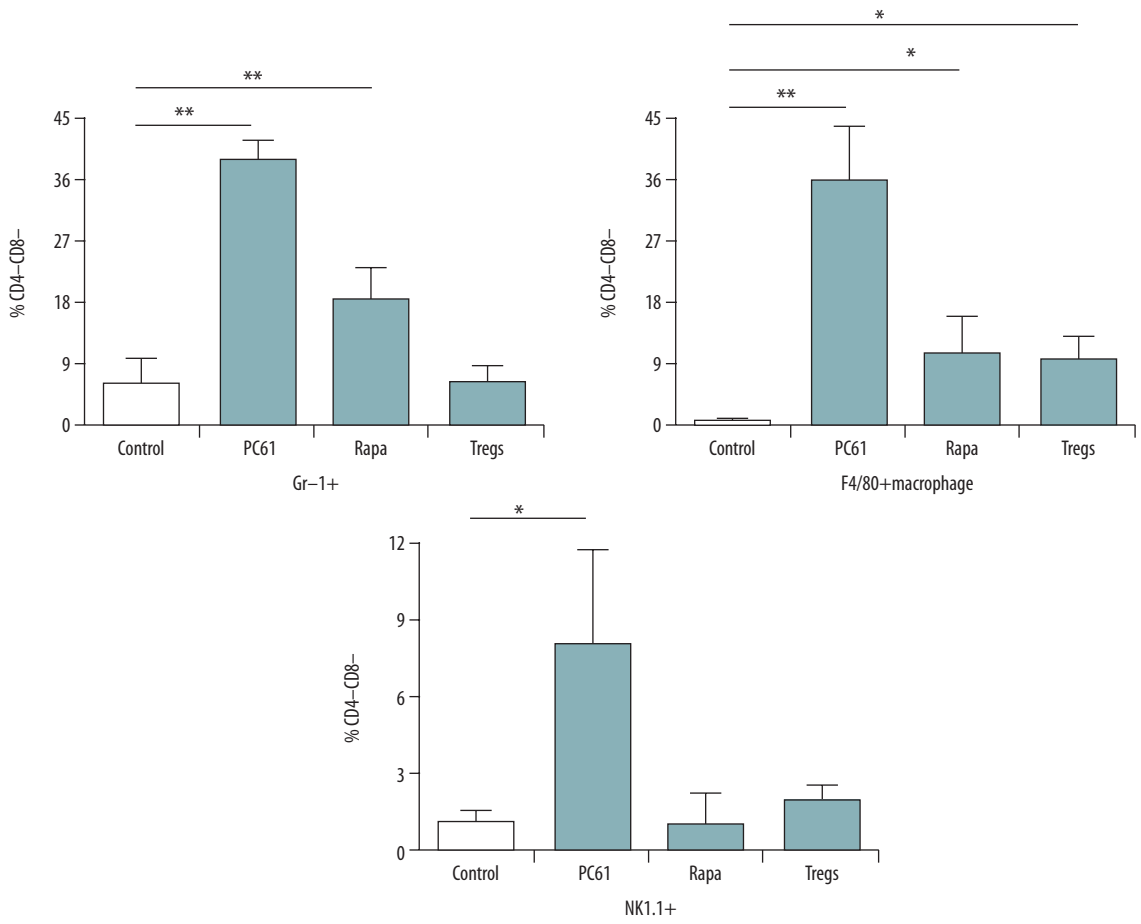

Figure 3. The frequency of Gr1+neutrophils, F4/80+ macrophage, NK1.1+ cells within cardiac graft of the transplant recipients. Graft-infiltrating these cells were collected for fluorochrome-labeling and subject to FACS analysis on day 3 after transplant. Statistical analysis was performed for the groups G1 (Cont), G2 (PC61), G3 (Rap), and G4 (Tregs).

that pre-sensitized immune condition of host could exaggerate prolonged cold ischemia-mediated injury of cardiac graft involving regulatory T cells [3]. It was found that Tregs are involved in the pre-sensitized condition. Nevertheless, the impact of exogenous adoptive transfer and endogenous induction of Tregs on innate immune cells was not clarified. Our study further unveiled that endogenous induction of Tregs by using rapamycin caused a significant decrease of frequency of splenic Gr1+ cells and a dramatic augmentation of frequency of Gr-1+ neutrophils and F4/80+ macrophages in the grafts. Exogenous adoptive transfer of Tregs resulted in an extreme decrease in frequency of splenic Gr1+ cells and a dramatic increase in frequency of Gr-1+ neutrophils and F4/80+ macrophages within grafts. Although the frequency of splenic NK1.1+ cells was not influenced irrespective of any treatment of PC61, adoptive transfer of Tregs, or rapamycin, administration of PC61 could dramatically increase frequency of NK1.1+ cells in the grafts.

Allogeneic immune responses cannot be effectively controlled partially because of the problems of innate, coagulopathic, and inflammatory responses. Innate cellular responses comprise of monocytes, macrophages, neutrophils, and natural killer cells [8]. Routinely, innate immune cells are recruited to the transplanted graft early after reperfusion, which promote the process of allograft rejection [9]. In a transplant scenario, it was found that regulatory T cells and innate immune cells had different susceptibilities to immunosuppressive treatment [10]. Previous studies showed that natural killer cells could exacerbate pro-inflammatory process and adaptive immune responses. In mice heart transplantation, allografts were rejected despite NK cell-deficiency since macrophages were obviously infiltrated into graft. These data implied that NK cells were capable of regulating monocyte or macrophage activation [11].

In previous pre-sensitized transplant scenarios, acute anti-donor antibodies-mediated allogeneic responses were normally

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studied [12], whereas natural killer, neutrophils, and macrophages populations were rarely highlighted for analysis. Indeed, presence of monocytes, macrophages, and natural killer cells impacts cardiac graft function in both xenotransplantation and allogeneic transplantation [13,14]. Recent study showed that deletion of the activating NK cell receptor NKG2D would potentiate rejection process of cardiac allografts [15]. Memory T cells may infiltrate transplanted grafts within hours after transplantation, and then grafts subjected to clinically relevant cold ischemic period are more susceptible to injury by these cellular infiltrates [14]. Thereafter, it is of great significance in investigating the interplay between regulatory T cells and NK cells, macrophages, and neutrophils. Whether these cells can interact with memory T cells is to be further studied.

Conclusions

Tregs depletion or Tregs induced by rapamycin or exogenous cell transfer could affect frequencies of both splenic and intragraft neutrophils, macrophages, and NK cells, but not splenic NK cells. Our findings advanced our understanding of sensitized transplant biology, which might shed light on enhancement of induction of sensitized transplant tolerance.

Conflict of interest

None.

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