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Combination of monoclonal antibodies with DST inhibits accelerated rejection mediated by memory T cells to induce long-lived heart allograft acceptance in mice

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ABSTRACT

Donor-reactive memory T cells mediated accelerated rejection is known as a barrier to the survival of transplanted organs. We investigated the combination of different monoclonal antibodies (mAbs) and donor-specific transfusion (DST) in memory T cells-based adoptive mice model. In the presence of donor-reactive memory T cells, the mean survival time (MST) of grafts in the anti-CD40L/LFA-1/DST group was 49.8 d. Adding anti-CD44/CD70 mAbs to anti-CD40L/LFA-1/DST treatment. The MST was more than 100 d (MST > 100 d). Compared with anti-CD40L/LFA-1/DST group, anti-CD40L/LFA-1/CD44/CD70/DST group notably reduced the expansion of memory T cells, enhanced the proportion of $CD4^+Foxp3^+$ regulatory T cells (Tregs) and suppressed donor-specific responses. Our data suggest that anti-CD40L/LFA-1/CD44/CD70 mAbs and DST can synergistically inhibit accelerated rejection mediated by memory T cells to induce long-lived heart allograft acceptance in mice.

1. Introduction

Inducing donor-specific tolerance is the ultimate goal of clinical organ transplantation, but a growing body of evidence exists to suggest that memory T cells may play a critical role in inhibiting allograft acceptance [1,2]. Transplant patients may develop alloreactive memory T cells after exposure to antigens during previous transplantations, blood transfusions, or pregnancies as well as due to continuous exposure to bacterial and viral pathogens [3–6]. Alloreactive memory T cells, especially virus-specific $CD8^+$ memory T cells, can cross-react with the allogeneic graft and cause rejection or threaten graft survival [1,5]. Notably, such primed cross-reactive T cells are resistant to classical co-stimulation blockade that are effective in controlling naïve T cells response [7]. T-cell depletion therapy used in clinical transplantation such as anti-CD3 (muromonab) or anti-CD52 (alemtuzumab) will increase the overall frequency of memory T cells, due both to the relative resistance of memory T cells as well as the likely conversion of naïve cells to memory T cells via homeostatic activation [8]. Hence the development of new treatments to effectively control memory T cells is a pressing therapeutic challenge.

From many early studies, we noted that CD44 and CD70 molecules are closely linked to memory T cells function. CD44 is an adhesion molecule that is expressed by most cells and mediates binding to the extracellular matrix and other cells via its ligand, glycosaminoglycan hyaluronic acid [9]. CD44 expression is upregulated on naïve T cells after activation via the T cell receptor, and high expression is maintained indefinitely on memory T cells [10]. Despite the wide usage of CD44 as a "memory marker", its function is still not well known [11]. We previously found anti-CD44 mAb treatment inhibited the function of $CD4^+$ memory T cells by decreasing IL-2 and IFN-γ expression and increasing IL-10 and TGF-β expression in the serum and the graft [12]. CD70 is a type II transmembrane glycoprotein belonging to the tumor necrosis factor family. Anti-CD70 mAb inhibited CD27 binding to CD70 on T cells, activated B cells and dendritic cells [13]. CD70 blockade prevented $CD8^+$ memory T cell-mediated rejection by diminishing the

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proliferation and activation of $CD8^+$ memory T cells *in vivo* but it had little effect on $CD4^+$ T cell function [14]. In light of these observations, CD44 and CD70 may well have unique properties, which prompted our interest in exploring whether the combination of anti-CD44 and anti-CD70 can synergistically inhibit the function of both $CD4^+$ and $CD8^+$ memory T cells.

In this study, used memory T cells-based adoptive mice model, we attempted to co-inhibit the 2 subsets of memory T cells and simultaneously block the activation of effector T cells to explore new treatment for controlling accelerated rejection. It was found that anti-CD44/CD70 mAbs in combination with anti-CD40L/LFA-1 mAbs significantly inhibited proliferation of memory T cells in *ex vivo* experiments but failed to induce long-lived heart allograft acceptance *in vivo*. Previous research has shown that DST has a synergistic effect with various monoclonal antibodies on inducing an early, robust, abortive expansion of the T cells that result in profound anergy, which has been remarkably successful in promoting permanent survival of heart and islet allografts [15,16]. Thus, DST was used to enhance the immunosuppressive effect of antibodies on combined effector CD4 and CD8-dependent host immune responses.

2. Materials and methods

2.1. Animals

Female C57BL/6 (H-2^b), BALB/c (H-2^d) and C3H (H-2^k) mice aged 8–12 weeks and weighing 20–25 g were used as recipients, donors or a third party source. All animals were maintained and bred in the specific pathogen-free facility.

2.2. Antibodies

All of the antibodies used for the treatment were purchased from Bio X Cell (West Lebanon, USA) including anti-CD40L (MR-1, M), anti-LFA-1 (M17/4, L), anti-CD44 (IM/7, W) and anti-CD70 (FR-70, F) mAbs and isotype antibodies. Antibodies for flow cytometric analysis, including FITC anti-CD3 (145-2C11), FITC anti-CD4 (GK1.5), FITC anti-CD8 (53-6.7), PE anti-CD44 (KM114), PECy5 anti-CD62L (MEL14), and their isotype controls were purchased from BioLegend (San Diego, USA). The mouse regulatory T cell staining kit was purchased from eBioscience (San Diego, USA).

2.3. Heart transplantation in memory T cells-based adoptive mice model

Full-thickness skin grafts were prepared from the lateral thoracic skin of the donor mice (BALB/c), cut into circular pieces ($\sim$1 cm^2 in area) and were engrafted onto the lumbar region of recipient mice (C57BL/6) that were so-called allo-primed. Four weeks after transplantation, T cells from spleens of allo-primed mice (that were allo-primed T cells) were harvested via nylon wool columns (Wako, Osaka, Japan). Single cell suspensions of allo-primed T cells were prepared, counted and the viability detected (>90% by trypan blue exclusion). The allo-primed T cells were then transferred to syngeneic recipient mice by a single intravenous (i.v.) injection (2×10^6 cells/recipient). On the day following the adoptive transfer, hearts from the BALB/c mice were transplanted to the neck vessels of the recipients [17]. After transplantation, the recipients received treatments of anti-CD40L, anti-LFA-1, anti-CD44, anti-CD70 mAbs and DST in various combinations (see Table 1 for experimental groups). The drugs were intraperitoneally (i.p.) administered on days 0, 2, 4, and 6 after surgery. Control mice were treated with isotype antibodies. Graft survival was monitored by daily palpation. Rejection was defined as the loss of palpable cardiac contractions.

Table 1
Various treatment combinations tested in this study.

Recipient mice	Treatment combinations[a]
Naïve	No treatment or M + L
Allo-primed T cells transferred	No treatment or M + L + W + F
Allo-primed T cells transferred plus DST[b]	No treatment or M + L or W + F or M + L + W + F

[a] Treatments consisted of 0.25 mg anti-CD154 (M)/0.25 mg anti-LFA-1 (L)/0.2 mg anti-CD44 (W)/0.5 mg anti-CD70 (F) mAbs in various combinations as indicated and were administered i.p. on days 0, 2, 4, and 6 after transplantation.
[b] DST: A total of 10×10^6 donor spleen cells were administered i.v. 1 day before the heart graft placement.

2.4. Extraction of lymphocytes from heart allografts

Sections from harvested cardiac allografts were minced with a sterile blade and incubated in 10 ml buffered saline with 2% BSA and 2 mg/ml collagenase at 37 °C for 2 h. The cells were strained through a 70 μm nylon cell strainer. Lymphocytes were isolated from these cells by EZ-Sep™Mouse lymphocyte separation medium (Dakewe Biotech Company, Shenzhen, China) with centrifugation for 20 min at 1600 rpm. After washing twice in RPMI 1640, the lymphocytes were resuspended in PBS with 10% fetal bovine serum.

2.5. Mixed lymphocyte reaction (MLR)

T lymphocytes isolated from the spleen of the recipient mice ($n = 3$ mice/group) using nylon wool columns (Wako, Osaka, Japan) were used as responder cells. Splenocytes obtained from BALB/c or C3H mice were treated with mitomycin (40 μg/ml, Amresco, Solon, USA) and used as stimulator cells. The stimulator cells with the responder cells at a 1:10 ratio were incubated at 37 °C for 72 h. Cell proliferation was measured using a bromodeoxyuridine (BrdU) cell proliferation assay kit (Chemicon, Billerica, USA). The measurements were performed in triplicate.

2.6. Cytokine enzyme-linked immunosorbent assays (ELISAs)

The culture supernatants from the mixed lymphocyte reactions were collected at 72 h, and sera from recipient mice ($n = 3$ mice/group) were isolated on days 5 and 45 post-transplantation. ELISAs were performed using commercially available kits (Yikesai Bioproduct Limited Company, Shanghai, China) to detect IL-2, IFN-γ, IL-10, and TGF-β according to the manufacturer's instructions. The measurements were performed in triplicate.

2.7. Quantitative real-time PCR (qRT-PCR)

Grafts were removed from recipients at days 5, 45 or 100 after transplantation, and the RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Reverse transcription and qRT-PCR were performed using ReverTraAce qPCR RT Kit (Toyobo, Osaka, Japan) and SYBR Green Realtime PCR Master Mix-Plus (Toyobo, Osaka, Japan), respectively. The StepOne Real-Time PCR System (ABI, Foster City, UK) was used. The expressions of *IL-2*, *IFN-γ*, *FasL*, *perforin*, *granzyme B*, *Foxp3*, *IL-10*, *TGF-β* were detected, and *β-actin* was used as a normalizing control. Calculation was performed using the $2^{-\Delta\Delta CT}$ method. Each reaction was carried out in triplicate. The primer sequences used for the qRT-PCR are from PrimerBank (http://pga.mgh.harvard.edu/primerbank/).