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Isatis tinctoria L. combined with co-stimulatory molecules blockade prolongs survival of cardiac allografts in alloantigen-primed mice

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ABSTRACT

Memory T cells present a unique challenge in transplantation. Although memory T cells express robust immune responses to invading pathogens, they may be resistant to the effects of immunosuppressive therapies used to prolong graft survival. In previous studies, we found that compound K, the synthesized analogue of highly unsaturated fatty acids from *Isatis tinctoria* L., reduced acute cardiac allograft rejection in mice (Wang et al., 2009 [1]). Here, we further investigated the effect of compound K on cardiac allograft rejection in alloantigen-primed mice. We found that compound K significantly inhibited $CD4^+$ and $CD8^+$ memory T cells proliferation in a mixed lymphocyte reaction (MLR). *In vivo*, compound K combined with anti-CD154 and anti-LFA-1 monoclonal antibodies (mAbs) significantly extended the survival time of heart grafts in alloantigen-primed mice with no obvious toxic side effects. Furthermore, our data suggests that compound K works by reducing the expression of both IL-2 and IFN-γ within the graft rather than enhancing expression of regulatory T cells (Tregs). Compound K can also inhibit the alloresponses of memory T cells, while increasing the proportion of $CD4^+$ memory T cells in the spleen of the recipients and significantly reducing the level of alloantibodies in the serum. Our study highlights the unique immune effects of compound K that may be further explored for clinical use in extending the survival of transplant grafts.

1. Introduction

Memory T cells are important components of the immune system that defend against invading pathogens. In adults, 40–50% of the T cells circulating in the peripheral blood have memory phenotypes [2,3]. Exposure to alloantigens during previous transplantation, blood transfusions, or pregnancy, as well as continuous exposure to bacterial and viral pathogens, and cross-reactivity with allogenic MHC molecules may result in the development of alloreactive memory T cells in transplant patients [4–6]. It has been reported that high frequencies of alloreactive memory T cells in the peripheral blood of transplant patients are associated with poor allograft outcomes [7]. Memory T cells are not sufficiently inhibited by the clinical first-line immunosuppressive agents and monoclonal antibodies [8], which makes them the most difficult barrier to overcome in order to extend the graft survival time of the initial and secondary transplants. Other evidence have shown that memory B cells and alloantibodies also play an important role in transplantation [9].

We previously extracted the highly unsaturated fatty acids from *Isatis tinctoria* L., an herb used in traditional Chinese medicine, and used them as templates to synthesize various similar compounds. In previous studies, we studied one of those agents, compound K (K), and found that when combined with tacrolimus it could significantly reduce acute cardiac allograft rejection in mice. The mechanism of action of this combined treatment was postulated to be through inhibition of the secretion of IL-2 and IFN-γ by lymphocytes, rather than the activation of Tregs [1].

In this study, we investigated the effect and potential mechanism of compound K on cardiac allograft rejection in alloantigen-primed mice. We used combinations of compound K with antibody-mediated blockade of co-stimulatory molecules (CD154 and LFA-1) to demonstrate potential synergistic effects on long-term survival of heart grafts in alloantigen-primed mice.

2. Objectives

The objectives of this study were three-fold. We wanted firstly to determine whether the compound K could reduce memory T cell

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proliferation in MLR; second, to determine whether compound K combined with anti-CD154 and anti-LFA-1 mAbs could reduce the accelerated rejection response in alloantigen-primed mice model; and third, to investigate the mechanism of action of compound K *in vivo*.

3. Materials and methods

3.1. Animals

Female C57BL/6 (B6) and BALB/c mice (8–12 weeks old) were purchased from Slac Laboratory Animal Co. Ltd. (Shanghai, China) and used as graft recipients and donors, respectively. Care and handling of animals were in accordance with the guidelines provided in the 'Guide for the Care and Use of Laboratory Animals' published by the U. S. Department of Health and Human Services.

3.2. Drugs and antibodies

The compound K, the analogue of highly unsaturated fatty acids from *Isatis tinctoria* L. was synthesized by associate professor Qin Qing from Guangxi Medical University (Guangxi, China). The following antibodies administered to the animals were produced from Bioexpress (West Lebanon): anti-CD154 (MR-1), anti-LFA-1 (M17/4), and their respective isotype controls. Antibodies used for FACS analysis were FITC anti-CD4 (GK1.5), FITC anti-CD8 (53-6.7), PECy5 anti-CD62L (MEL-14) and PE anti-CD44 (IM7), and their isotype controls (Biolegend, USA). Murine regulatory T cells were labeled using the Mouse Regulatory T cell staining kit from eBioscience (USA).

3.3. Murine cardiac transplantation model

Full-thickness BALB/c trunk skin tissues (circular pieces, diameter = 1.2 cm) were engrafted onto the lumbar region of B6 mice, and recipients that had rejected BALB/c skin 4 weeks after transplantation were defined as alloantigen-primed mice. Hearts from the donor mice were transplanted to the neck vessels of the alloantigen-primed B6 recipients using a non-suture cuff technique as described previously [10]. Graft survival was monitored by palpation (twice daily) and body weight was recorded daily for consecutive 15 days. B6 mice were treated with anti-CD154 (0.25 mg) + anti-LFA-1 (0.1 mg), compound K (10 mg/kg/day) or anti-CD154 (0.25 mg) + anti-LFA-1 (0.1 mg) + compound K (10 mg/kg/day). The antibodies were administered on the day of transplantation and 3 more times every alternate day. Compound K was given on days 0–10 post-transplantation, and the control group was given normal saline only.

3.4. Isolation of memory T cells

About 4 weeks after skin transplantation, the $CD4^+$ or $CD8^+$ memory T cells were autoMACS and FACS-purified from the spleens of the recipient mice. $CD4^+$ memory T cells were isolated by using the MagCellect Mouse Memory $CD4^+$ T Cell Isolation Kit (cat. no. MAGM206, R&D, USA). Briefly, splenocytes were incubated with a Biotin-antibody cocktail which targeted the unwanted cells, and then with microbead-conjugated anti-biotin mAb. The cell suspension was placed in magnetic field of a MACS separator. The supernatant in the tube is the final cell fraction containing the enriched $CD4^+$ Tm cells. Total $CD8^+$ T cells were also first isolated from spleen cells by using the MagCellect Mouse $CD8^+$ T Cell Isolation Kit (cat. no. MAGM 203, R&D, USA). These isolated $CD8^+$ T cells were then incubated with anti-CD8-FITC, anti-CD62L-PECy5 and anti-CD44-PE Ab and were sorted by FACS after gating on the $CD8^+CD44^{high}CD62L^{-/low}$ population. The purities of these cells were typically greater than 90% for $CD4^+$ memory T cells and greater than 95% for $CD8^+$ memory T cells detected by flow cytometry; the viability of these cells was greater than 90% detected by trypan blue staining.

3.5. Mixed lymphocyte reaction (MLR)

Memory T cells isolated from the spleen of the recipient mice using nylon wool columns (Wako, Japan) were used as responder cells. Spleen cells obtained from the BALB/c mice were used as stimulator cells. The responder cells (5×10^5 cells) were cultured in 96-well plates in the presence of stimulator cells (5×10^4 cells, pre-treated with mitomycin C, 40 μg/ml, Amresco, USA) in 200 μl RPMI 1640 supplemented with 10% FBS, penicillin, and streptomycin, and incubated at 37 °C in a 5% CO_2 humidified atmosphere for 72 h. Cell proliferation was measured using a bromodeoxyuridine (BrdU) cell proliferation assay kit (cat. no. 2750, Chemicon, USA). The measurements were performed in triplicates.

3.6. Pathological studies

Heart grafts ($n = 3$) were removed on day 5 post-transplantation. Part of each graft was used for quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), and the rest was used for histological evaluation. Paraffin-embedded transventricular tissue sections (5 μm thick) were stained with hematoxylin and eosin (H&E). Graft rejection was graded on the basis of the extent of infiltration and the anatomical localization of inflammatory cells, according to the International Society of Heart and Lung Transplantation (ISHLT) standard [11,12]. Briefly, heart tissue was scored as follows: 0 = no damage; 1 (mild) = evidence of interstitial edema and focal necrosis; 2 (moderate) = graft displayed diffuse myocardial cell swelling and necrosis; 3 (severe) = necrosis with presence of contraction bands and neutrophil infiltrate, and 4 (highly severe) = widespread necrosis with presence of contraction bands, neutrophil infiltrate and hemorrhage.

3.7. Quantitative real-time reverse transcription-polymerase chain reaction

Grafts were removed from the recipients on day 5 after transplantation, and the RNA was extracted using Trizol (Invitrogen, USA). Reverse transcription and PCR were performed using ReverTra Ace® qPCR RT Kit (code no. FSQ-101) and SYBR® Green Realtime PCR Master Mix -Plus- (code no. QPK-212, 212T) (Toyobo, Japan), respectively. Analysis of the data was performed using the StepOne Real-Time PCR System (ABI, UK). β-actin was used as a normalizing control, and each reaction was performed in triplicates. The primer sequences for qRT-PCR are: β-actin forward 5′-CATCCGTAAAGACCTCTATGCCAAC-3′, and reverse 5′-ATGGAGCCACCGATCCACA-3′; IFN-γ forward 5′-CGGCACAGTCATTGAAAGCCTA-3′, and reverse 5′-GTTGCTGATGGCCTGATTGTC-3′; IL-2 forward 5′-GGAGCAGCTGTTGATGGACCTAC-3′, and reverse 5′-AATCCAGAACATGCCGCAGAG-3′; IL-10 forward 5′-GACCAGCTGGACAACATACTGCTAA-3′, and reverse 5′-GATAAGGCTTGGCAACCCAAGTAA-3′; Foxp3 forward 5′-CAGCTCTGCTGGCGAAAGTG-3′, and reverse 5′-TCGTCTGAAGGCAGAGTCAGGA-3′; TGF-β forward: 5′-TGACGTCACTGGAGTTGTACGG-3′, and reverse 5′-GGTTCATGTCATGGATGGTGC-3′.

3.8. Microlymphocytotoxicity test (MLC)

The MLC test was performed by adding the following reagents to each well of a 96-well plate: 5 μl mineral oil, 1 μl serum from the recipient mice, 1 μl guinea pig serum, and 1 μl of a BALB/c spleen cell suspension (2×10^3 cells/μl). After incubation at 22–25 °C for 45 min, the cells were stained using the Cell Viability Assay Kit (Beyotime Institute of Biotechnology, China) and the apoptosis level was calculated by counting random visual fields. In negative control wells, 1 μl of serum from naive mice was used instead of that from experimental groups. Each reaction was performed in triplicates.