ORIGINAL ARTICLE

Short-term MyD88 inhibition ameliorates cardiac graft rejection and promotes donor-specific hyporesponsiveness of skin grafts in mice

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SUMMARY

Recognition of evolutionarily conserved ligands by Toll-like receptors (TLRs) triggers signaling cascades in innate immune cells to amplify adaptive immune responses. Nearly all TLRs require MyD88 to transduce downstream signaling. MyD88 deficiency has been shown to promote the allograft acceptance in mice. However, direct evidence for therapeutic potential of MyD88 inhibitors remains lacking. Herein, we used a MyD88 inhibitor, namely ST2825, to explore its therapeutic potential and mechanisms in fully allogeneic skin and heart transplant models. Phenotypic maturation of dendritic cells stimulated by TLR ligands was alleviated by ST2825 in parallel with reduced T-cell proliferation *in vitro*. A short-course treatment with ST2825 significantly prolonged cardiac graft survival (mean survival time = 18.5 ± 0.92 days vs. 7.25 ± 0.46 days). ST2825-treated group had significantly reduced proinflammatory cytokines in allografts compared with control group. ST2825 combined with anti-CD154 induced long-term skin allograft acceptance in about one-third of recipients (>100 days). ‘Skin-tolerant’ recipients showed attenuated donor-specific IFN-γ responses, intact IL-4 responses, and compromised alloantibody responses. We conclude that MyD88 inhibitor ST2825 attenuates acute cardiac rejection and promotes donor-specific hyporesponsiveness in stringent skin transplant models. The direct evidence suggests that pharmacological inhibition of MyD88 hold promising potential for transplant rejection.

Transplant International 2016; 29: 941–952

Key words

cardiac transplantation, dendritic cells, donor-specific hyporesponsiveness, MyD88, skin transplantation

Received: 28 April 2015; Revision requested: 5 June 2015; Accepted: 25 April 2016; Published online: 14 June 2016

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doi:10.1111/tri.12789

Introduction

Emerging strategies to ameliorate transplant rejection have been focused on targeting innate immunity in recent years [1–3]. The innate immune system rapidly reacts with stress factors and microorganisms through evolutionarily conserved mechanisms. Toll-like receptors (TLRs) are well-characterized innate immune receptors that broadly recognize conserved components from both self and nonself. During the transplant procedure, large amounts of TLR ligands are inevitably released as damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) in colonized organs [4–6]. Overactivated innate immune cells, especially dendritic cells (DCs), trigger specific clonal expansion of adaptive immune cells to attack the allografts, presenting formidable barriers to allograft acceptance. On the contrary, antigen-primed T cells undergo proinflammatory or tolerogenic responses, partly due to the activation status of DCs with which they make contact [7–9]. Thus, targeting components of innate immune signaling, such as TLR signaling, holds promising translational potential in transplant settings.

Myeloid differentiation primary response gene 88 (MyD88) is an intracellular adaptor protein exploited by nearly all TLRs to initiate their pathways. MyD88-mediated signaling cascade ultimately results in the upregulation of a broad range of proinflammatory molecules [10]. The importance of MyD88-mediated TLR signaling in transplant rejection has been recognized from bench to bedside. In mice models, it has been validated that sole MyD88 deficiency promotes a permanent acceptance of kidney allografts [11]. In contrast, skin allografts that are nonsterile and highly resistant to conventional costimulation blockade have been ascribed to an excessive TLR activation through MyD88 pathway [12,13]. Molinero *et al.* have reported that cardiac transplant tolerance could be reversed by transferring Langerhans cells from the same donors' skin grafts [14]. Tolerance resistance via TLR activation was dependent on increased secretion of type 1 interferon and IL-12 by antigen-presenting cells, reduced regulatory T cells, and enhanced proliferation of donor-specific effector $CD4^+$ and $CD8^+$ T cells [15,16]. In clinical settings, it has been revealed that TLRs were among the most highly upregulated gene transcripts in primary allograft dysfunction within 72 h following lung transplantation [17,18]. As we know, primary lung graft dysfunction is significantly positively correlated with poor long-term prognosis [18]. In addition, higher levels of TLR 4 mRNA have also been associated with primary kidney graft dysfunction [19]. Therefore, the intensity of TLR signaling largely determines the feasibility of tight control of rejection responses for better long-term graft outcomes.

However, data on therapeutic roles of MyD88 inhibitors remain lacking in transplantation models. Min *et al.* have reported that blocking the expression of MyD88 with siRNA alone could not promote the cardiac allograft survival [3]. This may be due to remnant MyD88 proteins in cytoplasm which cannot be suppressed at transcription level by siRNA. MyD88 activity requires the homodimerization mediated by BB loop. One peptidomimetic, ST2825, has been designed to specifically interfere with the homodimerization of MyD88 by targeting BB loop [20]. Herein, we exploit ST2825 in cardiac and skin allograft mouse models to confirm the roles of MyD88 inhibition in transplantation. Our results showed that pharmacological inhibition of MyD88 has novel implications for the treatment of transplant rejection.

Materials and methods

Mice

C57Bl/6 ($H\text{-}2^b$) mice, C3H ($H\text{-}2^k$), and BALB/c ($H\text{-}2^d$) (males, age: 8–12 weeks; weight: 20–25 g) were purchased from Beijing HFK Bioscience Company (Beijing, China). $MyD88^{-/-}$ BALB/c ($H\text{-}2^d$) mice were kindly provided by Dr. Maria-Luisa Alegre (University of Chicago, IL, USA). The protocol of genotyping and animal breeding was performed according to the procedures published previously [21]. All experimental procedures on animals used in this study were performed under a protocol approved by the Institutional Animal Care and Use Committee at the Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

Synthesis of CpG 1826 and MyD88 inhibitor ST2825

CpG oligodeoxynucleotide 1826 (CpG) was synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). The sequence used is as follows: 5′ TCC ATG ACG TTC CTG ACG TT 3′ (underlined bases were phosphorothioated). The peptidomimetic compound ST2825 was kindly provided by Dr. Vito Ruggiero (Department of Public Health and Cell Biology, University of Rome 'Tor Vergata', Rome, Italy) and was synthesized at Sigma-Tau Industrie Farmaceutiche Riunite S.p.A, Pomezia, Italy, as previously described [20].

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Propagation of bone marrow-derived DCs

C57Bl/6 bone marrow-derived DCs (BMDCs) were prepared as described previously [22]. Briefly, the bone marrow cells were flushed from the femurs and tibias of C57Bl/6 mice. The cells were treated with red blood cell lysis solution (Beyotime, Shanghai, China), washed, and cultured in complete RPMI 1640 medium supplemented with GM-CSF (10 ng/ml; PeproTech, Rocky Hill, NJ, USA) and IL-4 (10 ng/ml; PeproTech). Nonadherent cells were removed 48 h later. Fresh medium containing GM-CSF/IL-4 was changed every 48 h. On day 6, loosely adherent BMDCs were harvested for subsequent experiments.

Electrophoretic mobility shift assay and Western Blot

Bone marrow-derived DCs (approximately 5×10^5 cells) were stimulated with LPS (200 ng/ml) for 3 h in the presence of ST2825 (0, 10, 20, 40, and 60 μM) or vehicle control (DMSO). Nuclear proteins were extracted from BMDCs with a Nuclear Protein Extraction Kit (Beyotime, Shanghai, China). Electrophoretic mobility shift assay (EMSA) was performed with the LightShift Chemiluminescent EMSA Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, protein–DNA complexes were generated by incubating 5 μg nuclear extracts with an NF-κB 3′-end biotinylated DNA probe (5′ AGTT GAGGGGACTTTCCCAGGC 3′) at room temperature for 20 min, then loaded onto polyacrylamide gel, and transferred to a nylon membrane. The DNA transferred on the membrane was then exposed to ultraviolet light for cross-linking. After incubation with streptavidin–horseradish peroxidase and chemiluminescent substrate, visualization was performed by exposure to X-ray film. In parallel, Western blot assays were performed to detect the nuclear p65 levels with histone H3 as the internal control. Nuclear protein was extracted for separation, blotting, and incubation with primary antibodies for NF-κB p65 protein (Ab-311, Signalway Antibody; Baltimore, MD, USA) and histone H3 (TDY055, TDY Biotech; Beijing, China). After incubation with horseradish peroxidase-conjugated secondary antibodies, the blots were visualized by X-ray film after incubation with an enhanced chemiluminescence (ECL) system.

FITC-dextran uptake assay

To assess the effects of ST2825 on phagocytic activity of BMDCs, FITC-dextran assay was performed with the modified protocols as described previously [23]. BMDCs were treated with graded levels of ST2825 (0, 10, 20, 40, and 60 μM) or vehicle control (DMSO) at 37 °C in 5% CO_2 for 12 h. FITC-dextran (Sigma Aldrich, Deisenhofen, Germany) was added into each well to incubate with cells for additional 45 min. Then, the cells were washed three times to remove the excess FITC-dextran for flow cytometry analysis.

Flow cytometry analysis

Maturation of BMDCs was stimulated by lipopolysaccharide (LPS) (200 ng/ml; Sigma Aldrich), or CpG (10 μg/ml) for 12 h, in the presence of ST2825 (40 μM) or vehicle control (DMSO). Flow cytometry was used to detect the surface staining of BMDC maturation markers CD80 and CD86 (eBioscience, San Diego, CA, USA). Allospecific antibody responses to donor-derived antigens were assessed by indirect staining. Briefly, BABL/c splenocytes (0.5×10^6) were incubated with Fc-blocking anti-mouse CD16/CD32 antibodies (2.4G2; BD Biosciences, San Diego, CA, USA) in PBS containing 2% fetal bovine serum (FBS) at 4 °C for 30 min. After wash, 1:20 dilutions of recipient serum at 3 weeks or 110 days post-transplant as indicated in the text were added for further incubation followed by washing. Finally, FITC-conjugated anti-mouse IgG (eBioscience) was added to incubate with the cells, and flow cytometry was performed after wash.

Mixed lymphocyte reaction

Single-cell suspensions were obtained from the lymph nodes of C57Bl/6 mice. The cells were then stained with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen, Carlsbad, CA, USA). BMDCs from BALB/c mice were pretreated with ST2825 at different concentrations in the presence of CpG (10 ng/ml) for 12 h. After washing, BMDCs were treated with mitomycin C (MMC, 50 μg/ml; stimulator cells) for 15 min and were used to stimulate the proliferation of T cells. CFSE-labeled lymphocytes (1×10^6) and BMDCs (2×10^5) were seeded into 48-well plates in complete RPMI 1640 medium (1 ml). Five days later, the cells were harvested and stained with allophycocyanin-conjugated CD3. The proliferation of CFSE-labeled T cells was analyzed by gating on $CD3^+$ cells.

Cardiac transplantation and ST2825 treatment

Mouse cervical cardiac transplantation was performed as described previously [24]. The day of the operation

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was recorded as day 0. As the solubility of ST2825 in water was not desirable, we employed carboxymethylcellulose (CMC) to promote its absorption *in vivo*. ST2825 suspended in 0.5% CMC was administered to experimental recipients by intraperitoneal injection (i.p.) from day 0 to day 6 (250 mg/kg/day). The complete cessation of cardiac contractility was defined as the observation endpoint.

Skin transplantation and tolerance induction

Full-thickness allogeneic skin grafts (approximately 1 cm^2) from the tails of donor mice were transplanted to the dorsal flank of recipient mice (C57Bl/6). For tolerance induction, the recipient mice were treated with ST2825 (suspended in 0.5% CMC, 250 mg/kg/day, i.p.) on days 0–3, 5, 7, 9, 11, 13, and 15 and/or anti-CD154 (MR1 clone, 200 μg/day, i.p.; Bio Express, West Lebanon, NH, USA) on days 0–3, 7, and 14.

Real-time PCR and histology

Cardiac grafts were harvested on day 3 for real-time PCR (RT-PCR) analysis, which was performed with the Quantitative SYBR Green PCR Kit (TOYOBO, Osaka, Japan) on the Rotor Gene 3000 (Corbett Research, Sydney, NSW, Australia). Cycling parameters were as follows: 45 cycles at 95 °C for 15 s, 56 °C (or 55 °C) for 15 s, and 72 °C for 20 s. Each PCR was performed in triplicate, and the PCR products were subsequently run on a 1.5% agarose gel to confirm the specificity of PCR. Fold changes were determined based on the calculations using the threshold cycle (Ct) of genes relative to the Ct of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers used are shown in Table S1. On day 5, cardiac grafts were obtained for histological examination with hematoxylin–eosin (HE) staining.

Enzyme-linked immunosorbent spot assay

The assays were performed according to the manufacturer's instructions (eBioscience). Briefly, 96-well polyvinylidene difluoride membrane enzyme-linked immunosorbent spot (ELISPOT) plates (Millipore; Billerica, MA, USA) were precoated with capture antibodies overnight. Spleen cells (1×10^6/well) were plated in complete RPMI-1640 with MMC-treated spleen cells (stimulator cells, 1×10^6/well) and incubated at 37 °C in 5% CO_2 for 36 h. Cytokines were detected using the 3-amino-9-ethylcarbazole peroxidase substrate kit. Spots were quantitated using the ImmunoSpot Analyzer (CTL Analyzers, Cleveland, OH, USA).

Adoptive transfer of treated BMDCs in MyD88$^{-/-}$ HY-mismatched skin transplantation

MyD88$^{-/-}$ male to female (BALB/c, *H-2*^{d}) HY-mismatched skin transplant tolerance model was established. BMDCs from wild-type BALB/c male mice were sorted by magnetic cell sorter cell-separating system (Miltenyi Biotec Inc., Bergisch Gladbach, Germany) (> 90% purity). The purified BMDCs were pretreated with 40 μM ST2825 or vehicle control (DMSO) for 12 h and then transferred to recipients (2×10^6 cells/mice) at the time of HY-mismatched skin transplantation.

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM). Difference between the groups was analyzed with one-way ANOVA followed by Bonferroni test for difference between groups. Difference in graft survival was analyzed by Kaplan–Meier curves and log-rank test. Mann–Whitney *U*-test was used to compare the difference in alloantibody mean fluorescence intensity (MFI) between the groups. Statistical analysis was performed using GRAPHPAD PRISM software version 5.01 (La Jolla, CA, USA). *P*-values <0.05 were considered statistically significant.

Results

ST2825 at therapeutic concentrations dose dependently inhibits the activation of NF-κB in BMDCs stimulated by LPS with intact phagocytic ability

NF-κB activation is one of the well-characterized molecular events in TLR signaling cascades. To investigate the influence of MyD88 inhibition on the activity of NF-κB, BMDCs stimulated by LPS were concurrently incubated with ST2825 at increasing levels (Fig. 1a). EMSA showed that ST2825 significantly inhibited NF-κB nuclear translocation in a dose-dependent manner, in parallel with the alteration in NF-κB p65 levels in nuclear extracts. To exclude the possible toxic effects of ST2825 on BMDCs, phagocytic activity was assessed by FITC-dextran uptake assays. The MFI of FITC-dextran was slightly reduced in the ST2825 (60 μM) group compared with the no-treatment group (*$P < 0.05$, Fig. 1b), whereas BMDCs treated with

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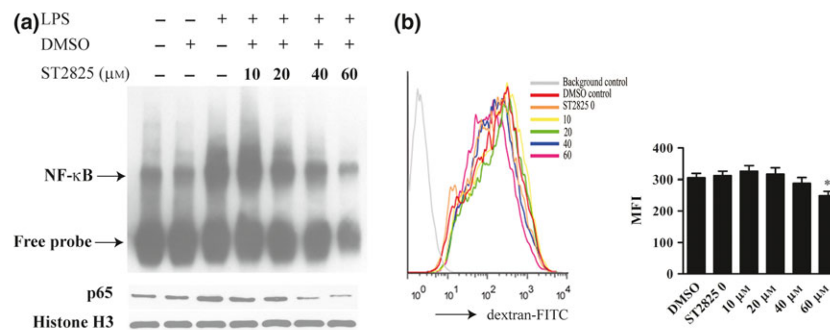

Figure 1 ST2825 inhibits LPS-stimulated NF-κB translocation in BMDCs without the apparent impairment in phagocytic activity. (a) BMDCs were incubated with LPS (200 ng/ml) in the presence or absence of ST2825 for 3 h. The levels of NF-κB nuclear translocation were assessed by EMSA (upper panel). NF-κB p65 levels were detected by Western blot in nuclear extracts (lower panel). Results are representative of three separate experiments. (b) FITC-dextran uptake assay was used to determine the phagocytic capacities of BMDCs treated by ST2825 or control. MFI is shown as mean ± SEM of triplicate wells from three independent experiments; $^*P < 0.05$, compared with DMSO control.

ST2825 at levels lower than 60 μM had a comparable phagocytic activity.

MyD88 inhibition prevents phenotypic maturation of BMDCs stimulated by TLR agonists

Phenotypic maturation of BMDCs is largely dependent on TLR engagement. LPS and CpG are typical components of microbial component triggering MyD88-dependent pathway. In addition, a portion of LPS/TLR4 signaling was MyD88 independent. Given that ST2825 could inhibit LPS-mediated NF-κB nuclear translocation, we sought to explore whether phenotypic maturation was impaired as well. Levels of CD80 and CD86 induced by CpG or LPS were markedly reduced by ST2825 treatment (Fig. 2a,b). These results indicate that MyD88 inhibition is potent enough to prevent the upregulation of costimulatory molecules on BMDCs stimulated by MyD88-dependent TLR agonists.

Inhibition of MyD88 in BMDCs prevents the activation of T lymphocytes

The effects of MyD88 inhibition on the priming capacity of BMDCs were further explored with CFSE dilution assay. Immature BMDCs stimulated by CpG were pretreated with ST2825 for 12 h and then washed three times before co-culture with lymphocytes. BMDCs pretreated with ST2825 (10 μM, 20 μM, and 40 μM) displayed a significantly reduced priming capacity compared with the vehicle control group ($CFSE^{dim}$ $CD3^+$ cells: 41.83% ± 1.39%, 36.13% ± 1.78%, 32.17% ± 1.62%, and 55.60 ± 1.93%, respectively; Fig. 3). These results support that ST2825 prevented the functional priming capacity of BMDCs stimulated by TLR agonists.

Short-course suppression of MyD88 alone promotes the survival of fully allogeneic cardiac allografts *in vivo*

It has been shown that a lack of MyD88 signaling both in donors and in recipients is very modest in protecting fully allogeneic cardiac allografts [25]. The therapeutic potential of ST2825 in fully allogeneic heart transplantation (BALB/c to C57Bl/6 mice) was therefore investigated. Remarkably, a short-course administration of ST2825 post-transplantation significantly prolonged the survival of cardiac allografts (MST = 18.5 ± 0.92 days vs. 7.25 ± 0.46 days, $P < 0.01$; Fig. 4a). In addition, lymphocyte infiltration and myocardial necrosis were reduced in the ST2825-treated group compared with controls (Fig. 4b). In the early stage of acute rejection (day 3), the expression levels of IL-1β, IL-6, and TNF-α mRNAs were several folds greater in the control (CMC) compared with that of ST2825 treatment: 4.41-, 2.06-, and 2.81-fold respectively (Fig. 4c). To determine the donor-specific responses, IFN-γ ELISPOT was performed on day 7. ST2825-treated group showed significantly reduced responses (Fig. 4d). These results demonstrate that the inhibition of innate immune by targeting MyD88 dampens the adaptive responses and prolongs the cardiac allograft survival.

A short-course administration of ST2825 combined with costimulation blockade promotes donor-specific hyporesponsiveness of skin allografts

Intrinsic microbe-harboring organs such as skin, lung, and intestine are highly resistant to tolerance induction. Activation of TLR signaling is considered to be the main molecular pathway executing resistance to tolerance in these organs [13,14]. We sought to explore the

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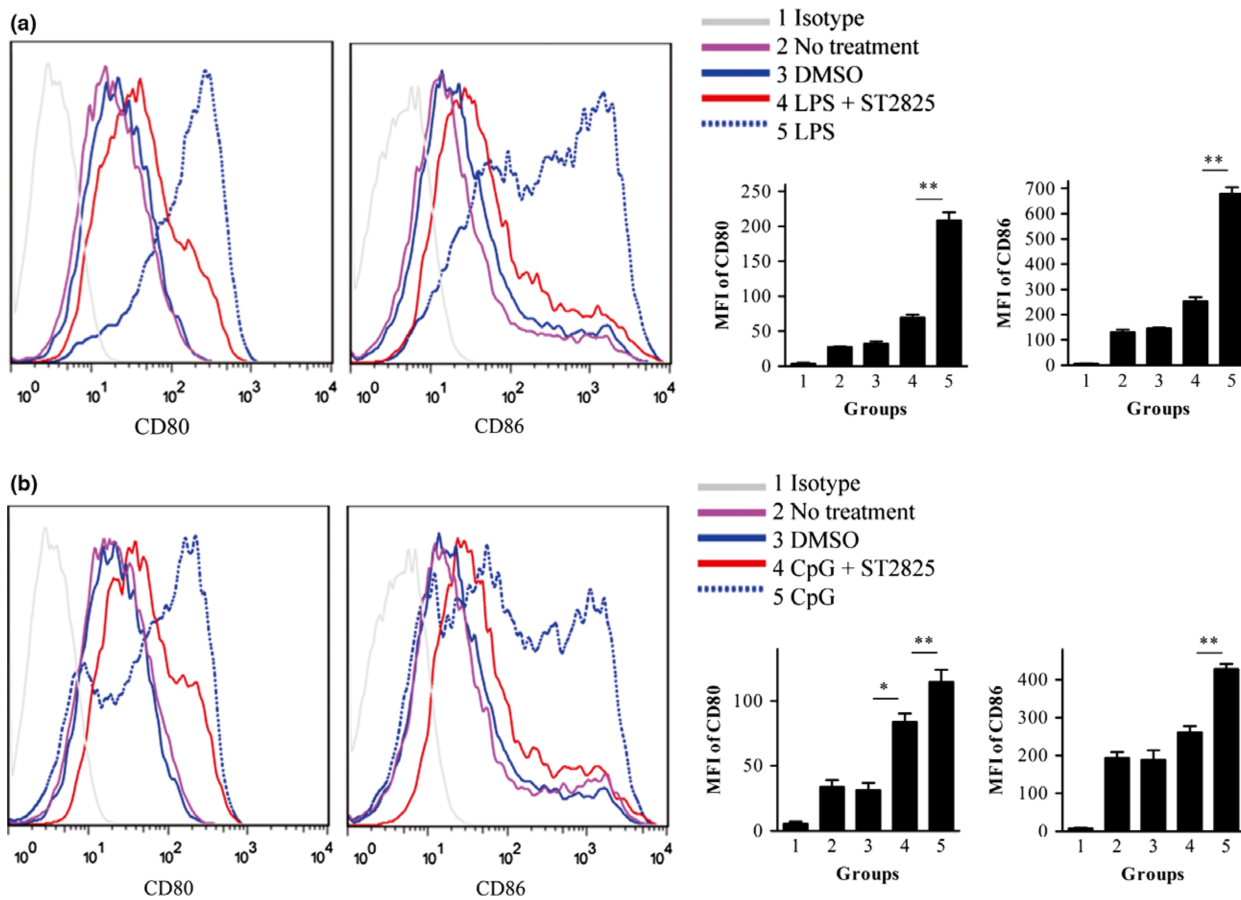

Figure 2 ST2825 interferes with phenotypic maturation of BMDCs stimulated by TLR agonists. BMDCs were stimulated with LPS (200 ng/ml) or CpG (10 μg/ml) for 12 h in the presence of 40 μM ST2825 or control. MFI of CD80 and CD86 on BMDCs were analyzed by gating on $CD11c^{+}$ cells. Data were shown as mean ± SEM (** $P < 0.01$; * $P < 0.05$).

potential of ST2825 in the induction of skin transplant tolerance (Fig. 5a). Surprisingly, treatment with ST2825 and anti-CD154 promoted permanent skin allograft acceptance (>100 days) in about one-third of the recipients (Fig. 5b). To molecularly characterize the tolerance state in these recipients, ELISPOT assays were performed to detect the secretion of proinflammatory cytokines IFN-γ and IL-4 (Fig. 5c–e). 'Skin-tolerant' recipients retained normal IL-4 responses, but exhibited significantly reduced IFN-γ secretion compared with the other two groups. To evaluate the alloantigen-specific IgG responses, serum from different recipients was collected at 3 weeks or on 110 days post-transplantation using a flow cytometric antibody binding assay (Fig. 5f). 'Skin-tolerant' recipients showed comparable MFI of anti-mouse IgG compared with the allogeneic recipients treated with ST2825, anti-CD154 (MR1), or the combination of ST2825 and MR1 at 3 weeks post-transplantation. All treated recipients had lower anti-mouse IgG levels than the untreated recipients. These results show that donor-specific hyporesponsiveness induced by ST2825 and anti-CD154 was associated with the Th2 immune deviation and reduced alloantibody responses.

Inhibition of MyD88 activity in DCs is critical for skin transplant tolerance

Toll-like receptors are mainly expressed on innate immune cells, such as DCs. We next sought to explore whether DCs were the target cells by MyD88 inhibition in the establishment of long-term skin acceptance. We employed a spontaneous skin transplant (male to female) model in $MyD88^{-/-}$ mice [26]. Previously, we have found that adoptive transfer of wild-type BMDCs to $MyD88^{-/-}$ female recipients prevented the establishment of HY-mismatched skin graft tolerance [27]. In this study, wild-type BMDCs were pretreated with ST2825 for 12 h and reintroduced into the $MyD88^{-/-}$ female recipient mice which were then transplanted with skin grafts from $MyD88^{-/-}$ male mice. All

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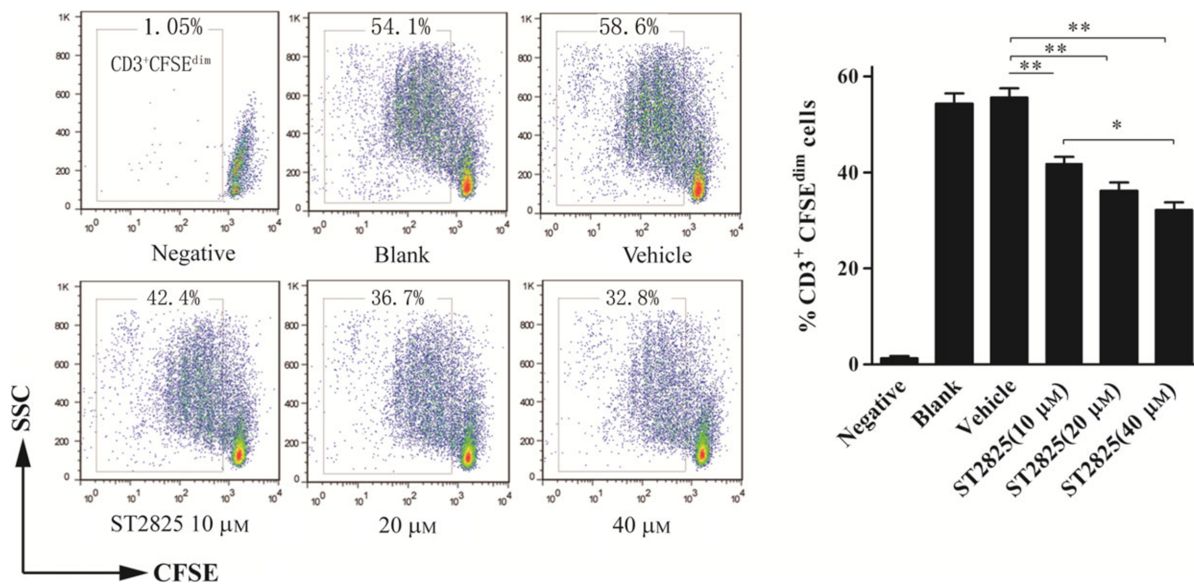

Figure 3 ST2825 suppresses alloantigen-priming capacities of TLR-stimulated BMDCs. BALB/c-derived BMDCs were stimulated with CpG (10 μg/ml) in the presence of ST2825 or DMSO control for 12 h. After wash, BMDCs were then co-cultured with CFSE-labeled lymphocytes from the lymph nodes of C57Bl/6. Groups with no BMDCs, untreated BMDCs, or DMSO-treated BMDCs were served as negative, blank, and vehicle control, respectively. Proliferating T lymphocytes with diluted CFSE were determined by gating on $CD3^+$ cells (**$P < 0.01$; *$P < 0.05$).

recipients that received wild-type or vehicle-treated BMDCs rejected the skin allografts. In contrast, recipients that received ST2825-treated BMDCs ($n = 9$) displayed a prolonged allograft survival (>100 days; Fig. 6). The data suggest that MyD88 inhibition in DCs promotes the establishment of skin transplant tolerance.

Discussion

Although innate immune system is not sufficient to elicit rejection without adaptive immunity, it creates inflammatory environment that augments the activation of adaptive responses. Under constant evolutionary pressure, organisms have shaped a limited set of pattern recognition receptors (PRRs) to recognize diverse molecules of microbial origin and DAMP released by dying or stressed cells [28,29]. TLRs are regarded as the major components of PRRs responsible for self/nonself discrimination. Current insights into fundamental elements in innate immune form the rationale that therapeutic strategies against these molecular events hold a great potential for improving transplant outcomes.

In this study, we provide translational evidence that a short-term treatment with a MyD88 inhibitor not only suppresses cardiac allograft rejection but also has powerful potential to induce long-term acceptance of highly immunogenic skin grafts. In contrast to MyD88 inhibition, MyD88 deficiency did not show profound protection on fully allogeneic cardiac grafts [25]. Effects caused by short-term MyD88 inhibition seem to be different from MyD88 deficiency, which may have undergone functional or developmental compensation through other PRRs. For instance, clinical patients deficient in MyD88 had compromised resistance to infection during childhood, whereas symptoms were spontaneously improved in adolescence [30]. Moreover, it has been shown that simultaneous deletion of Toll-interleukin 1 receptor (TIR) domain-containing adaptor inducing IFN-β (TRIF) and MyD88 prolongs the survival of skin allografts compared with recipients with MyD88 deficiency alone [31]. Therefore, it is possible that TRIF pathway becomes predominant for cardiac rejection responses in $MyD88^{-/-}$ models. Nevertheless, lung and kidney recipients with LPS-hyporesponsive TLR4 polymorphism tended to have reduced acute rejection episodes [32–34]. Data on the correlation between MyD88 polymorphisms with transplant outcome remain unknown.

Both ST2825 and anti-CD154 exerted protective effects on cardiac allografts, but either alone did not prolong the survival of skin allografts. It has long been observed that nonsterile organs are among the most difficult grafts to be induced transplant tolerance [13,18]. Our studies provide the direct evidence that MyD88 inhibition can bring practical benefits to long-term survival of skin grafts. Although we found that long-term acceptance of skin grafts was broken by retransplanting donor-derived skin (unpublished

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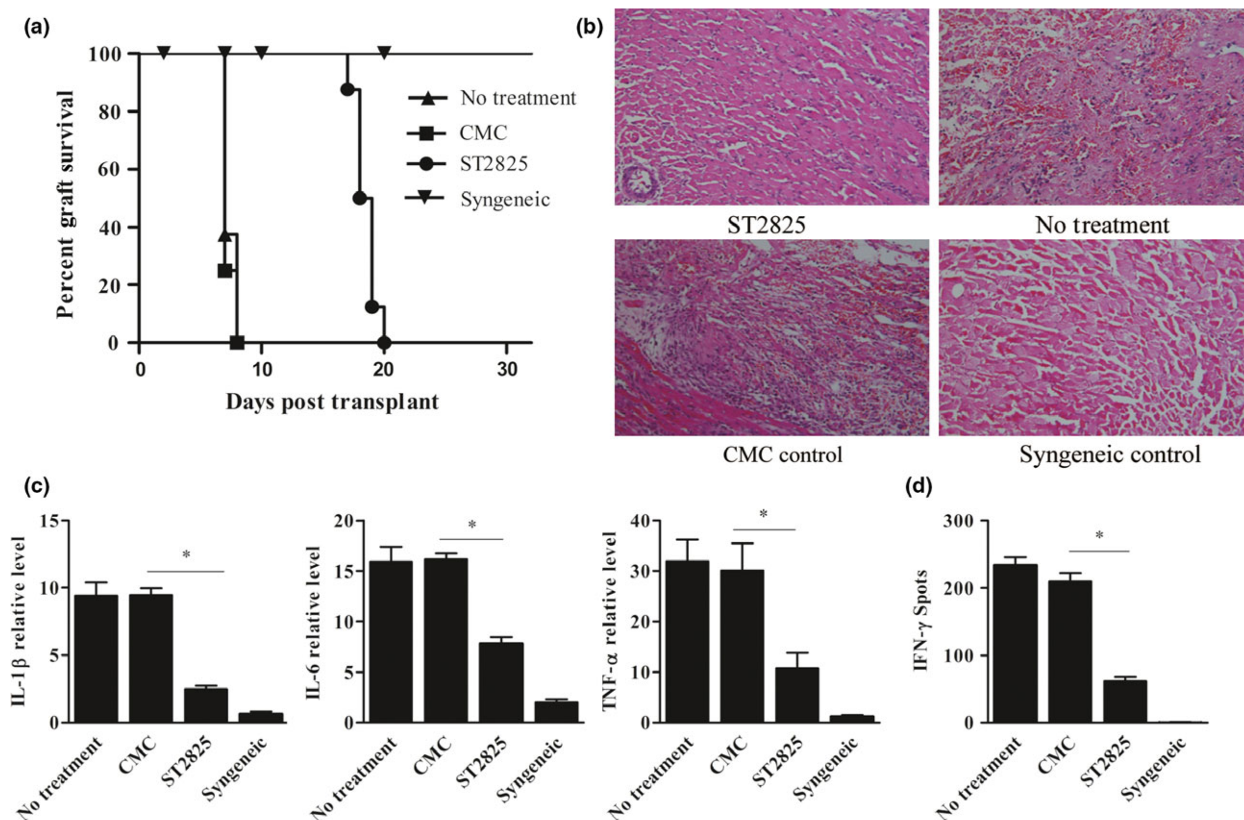

Figure 4 A short-course administration of ST2825 prolongs the survival of fully allogeneic cardiac grafts. (a) Survival curve of cardiac allografts was plotted as function of days post-transplantation (B/c, BALB/c; B/6, C57Bl/6). (b) HE-stained sections of grafts removed on day 5 are shown (× 200). (c) The relative levels of IL-1β,IL-6, and TNF-α mRNA in grafts on day 3 were measured by qRT-PCR (*, $P < 0.05$). (d) Donor-specific responses in recipients were analyzed by IFN-γ ELISPOT on day 7 (*$P < 0.05$).

results), our findings still have implications for therapies directed against MyD88 for better outcome of nonsterile grafts. However, our strategies aiming to induce skin transplant tolerance only promoted the long-term allograft acceptance in a proportion of the recipients, which seems to be inconsistent with the observation that 100% MR1-induced skin tolerance was seen in MyD88$^{-/-}$ recipients. Several reasons may account for this discrepancy. Firstly, the levels of ST2825 in nonvascularized skin allografts are supposedly much lower than those in circulation, which may spare a large amount of Langerhans cells within the allografts. Similarly, compromised skin tolerance induced by MR1 was observed in MyD88$^{-/-}$ recipients transplanted with wild-type skin grafts [13]. Secondly, we cannot exclude the possibility that the dosage of ST2825 used is not powerful enough to fully inhibit MyD88 especially at the early stages of skin transplantation. In addition, the solubility of ST2825 in water is not so excellent that we have to suspend it in CMC medium to administer into the recipients. If these obstacles are overcome in future studies, we may succeed in increasing the rates of skin tolerance and establishing more potent tolerance state.

To address the mechanism by which MyD88 activation in DCs contributes to graft rejection, we used a MyD88$^{-/-}$ HY-mismatched skin transplant tolerance model. In the model, tolerance reversed by the adoptive transfer of wild-type DCs could be restored by pretreatment with ST2825, indicating the importance of Myd88 signaling of DCs in skin transplant rejection. Interestingly, a previous study has shown that the adoptive transfer of epidermal DCs reversed the spontaneous cardiac tolerance [14]. These findings indicate that inflammatory environment within skin grafts facilitates highly responsive DCs to prime adaptive immune responses. Moreover, skin-specific alarmin, haptoglobin, has recently been demonstrated to be involved in skin rejection responses through MyD88-dependent pathway [35]. It should be pointed out that TLRs and MyD88 are also expressed in a broad range of cells, including B cells, macrophages, T lymphocytes, and other nonimmune cells [36–39]. Recently, it has been uncovered that selectively deficiency of MyD88 in B cells completely abolishes antibody-mediated inflammation [40]. ST2825 also has been proved to inhibit the secretion of autoantibodies from human B cells

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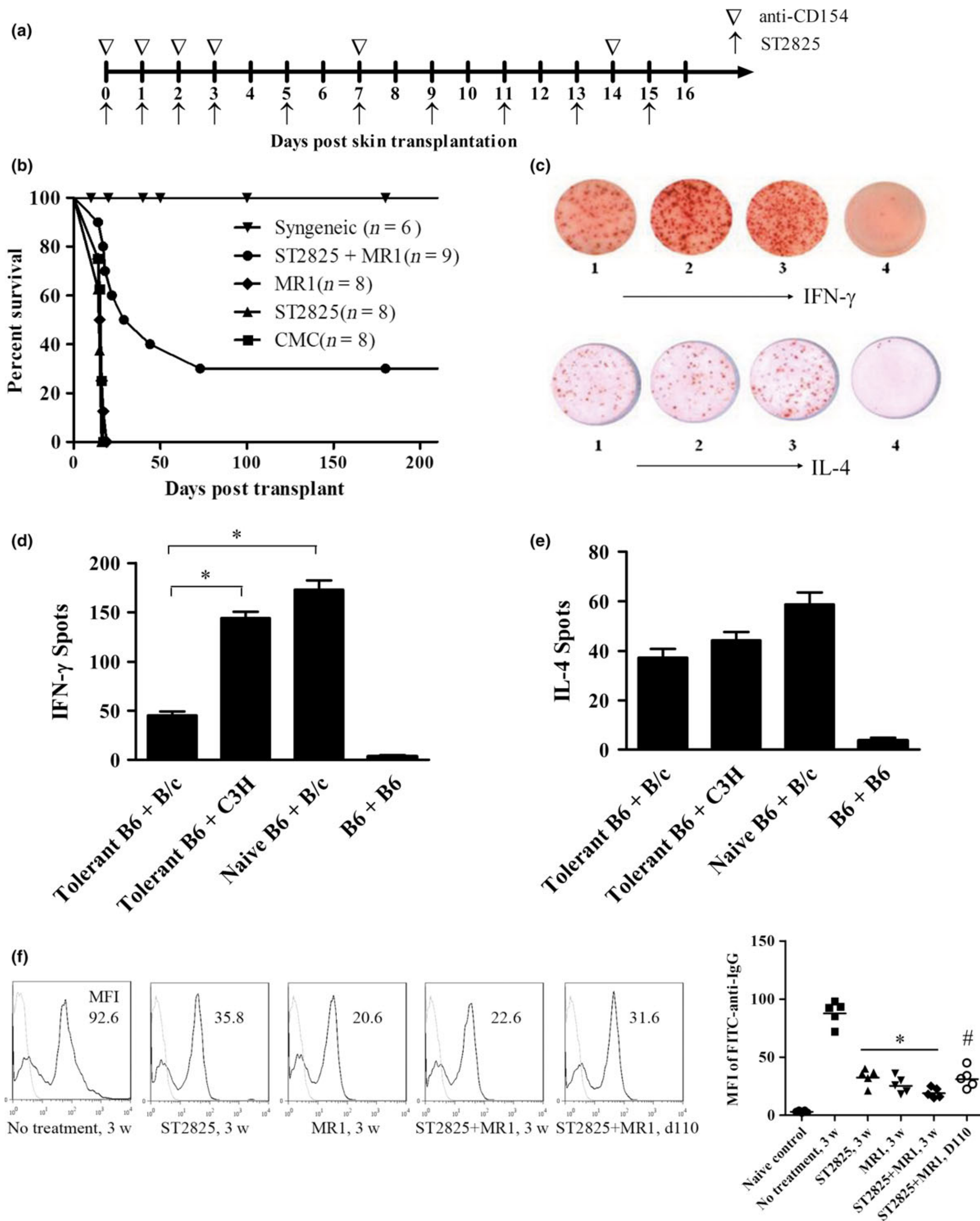

Figure 5 ST2825 combined with anti-CD154 induces long-term skin allograft acceptance due to compromised cellular and humoral responses. (a) A schematic diagram is shown to illustrate the tolerogenic regimen within the 15 days after allogenic skin transplantation. (b) The survival time of skin grafts was shown. (c) ELISPOT assays performed with splenic cells harvested from tolerant recipients on day 110 post-transplantation (n = 3), which were stimulated with splenic cells from donor (BALB/c, Lane 1), third-party (C3H, Lane 2), or syngeneic (C57Bl/6, Lane 4) mice. Splenic cells from naïve C57Bl/6 stimulated with naïve BALB/c mice-derived splenic cells were served as positive control (Lane 3). One representative plot of three independent experiments is shown. The quantitative numbers of spots for IFN-γ (d) and IL-4 (e) are shown (*, $P < 0.05$). (f) Comparison of donor-specific anti-IgG titers between 'skin-tolerant' recipients and other recipients or control. The MFI levels were lower in the treated recipients at 3 weeks post-transplant than in the untreated recipients (n = 5 per group. *, Mann–Whitney U-test, $P < 0.05$). The 'skin-tolerant' recipients maintained lower anti-IgG levels on day 110 post-transplantation than the untreated recipients (#, Mann–Whitney U-test, $P < 0.05$). Naïve C57Bl/6 mice with no treatment were served as negative control (gray lines).

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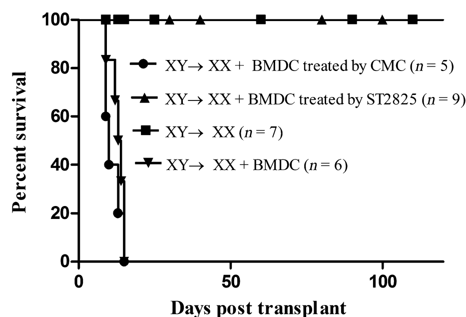

Figure 6 MyD88$^{-/-}$ BALB/c HY-mismatched skin transplant tolerance is reversed by adoptive transfer of wild-type BMDCs, whereas tolerance is restored by ST2825 pretreatment of BMDCs before transfer. MyD88$^{-/-}$ BALB/c (*H-2^d*) female mice received skin allografts from MyD88$^{-/-}$ BALB/c male mice. At the time of skin transplantation, purified BMDCs from wild-type male BALB/c mice pretreated by ST2825 or vehicle were intravenously injected into the MyD88$^{-/-}$ female recipients. Survival curves of the skin grafts are shown.

stimulated by TLR 9 [41]. In our model, further studies are required to ascertain whether ST2825 treatment affects the antibodies production against the grafts. As MyD88$^{-/-}$ T cells have no intrinsic deficiency for the maximal proliferation responses [12], it seems that DCs while not T cells are the main target cells of ST2825 for graft acceptance. ELISPOT assays indicated that low responses to donor antigens remain in the recipients with long-term skin graft acceptance (>100 days). Hence, donor-specific low responsiveness rather than tolerance was established. Although Th2 immune deviation is not necessarily associated with long-term graft acceptance [42], intact IL-4 responses have been observed in these recipients. In clinical settings, infection presents the major caveat resulting in the destabilization of established tolerance state, which may be associated with TLR activation [43]. A previous study has shown that infection with *Listeria monocytogenes* could reverse the established cardiac allograft tolerance in mice with the involvement of several mechanisms, including MyD88-dependent pathway and synergistic effects of IL-6/IFN-α [44,45]. It warrants further studies to investigate the roles of MyD88 inhibition in the maintenance of transplant tolerance.

The current paradigm incorporates innate immune as an important arm in transplant rejection responses; nevertheless, adaptive immunity should also be taken into account. Spontaneous skin transplant tolerance established in HY-mismatched MyD88$^{-/-}$ mice implicates vital roles of T-cell repertoire size against alloantigens. In wild-type mice, anti-CD154 treatment has been shown to induce the abortive expansion of effector T-cell pools in transplantation [46]. Minimal conditioning regimens involving T-cell depletion have been shown promising results in achieving long-term allograft acceptance [47,48]. These strategies combined with MyD88 inhibition might have synergic effects on tolerance induction for nonsterile grafts. Although several costimulatory signals participate in the activation of T cells and targeting these signaling pathways remains challenging [49], successful instances have also shed light on this strategy in clinical settings [50]. Based on our study, the combination of costimulation blockade with MyD88 inhibition may bring further benefits for allograft survival. Alternatively, currently available immunosuppressants combined with MyD88 inhibitors might be used as measures to obtain better long-term outcomes. For instance, the primary dysfunction of lung allografts has significantly poor outcomes in which innate immunity plays important roles [18]. It remains largely unknown whether MyD88 inhibitors are beneficial to improve the short-term and long-term outcomes of the highly immunogenic lung grafts.

In conclusion, our results demonstrate that pharmacological inhibition to MyD88 not only suppresses acute rejection but also promotes the long-term acceptance of fully allogeneic skin grafts. Strategies introducing MyD88 inhibition may render transplantation tolerance more broadly achievable.

Authorship

WH: participated in research design, performance of research, data analysis, and writing of the article. LZ and CL: participated in research design, performance of research, and data analysis. SL, ZD, ZF and FM: participated in performance of research. ZC and PZ: participated in the research design and review of the manuscript.

Funding

This study was funded by grants from the National High-Tech Research and Development Program (Program 863) of the Ministry of Science and Technology of China (grant 2012AA021010) and the National Natural Science Foundation of China (grants 81072442, 81471588, and 81102260).

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Acknowledgements

We express our gratitude to Dr. Vito Ruggiero for his kind help throughout the study. We also thank Professor Maria-Luisa Alegre for providing MyD88$^{-/-}$ mice.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Primers used for RT-PCR.

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