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Peptide Tk-PQ induces immunosuppression in skin allogeneic transplantation via increasing Foxp3$^+$ Treg and impeding nuclear translocation of NF-κB

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ABSTRACT

Solid organ transplantation is used as the last resort for patients with end-stage disease, but allograft rejection is an unsolved problem. Here, we showed that Tk-PQ, a peptide derived from trichosanthin, had an immune-suppressive effect without obvious cytotoxicity in *vitro* and in a mouse skin allo-transplantation model. In *vitro*, treatment of Tk-PQ administrated type 2 T helper cell (Th2)/regulatory T-cell (Treg) cytokines, and increased the ratio of CD4$^+$CD25$^+$Foxp3$^+$ Treg by repressing the PI3K/mTOR pathway. In addition, Tk-PQ decreased NF-κB activation to downregulate pro-inflammatory cytokines. Tk-PQ treatment in the mouse skin transplantation model also caused the similar molecular and cellular phenotypes. Furthermore, Tk-PQ enhanced the suppressive function of Treg by increasing Foxp3 expression, and substantially improved allograft survival. These finding demonstrate that Tk-PQ has the potential to be used in clinical allogeneic transplantation.

1. Introduction

Since the first skin autograft-transplantation by Carl Bunger in 1823, solid organ transplantation has often been used as the last resort for patients with advanced lung disease, end-stage kidney disease, and liver cirrhosis who cannot be cured by drug or other methods (Afonso et al., 2015; Meirelles et al. 2015). Nevertheless, graft rejection and complications are the major factors contributing to the poor survival of transplantation patients (Isoniemi et al., 1992; Hopkins et al., 2002; Christie et al., 2010; Boor and Floege, 2015). Graft rejection was considered to be mainly associated with the immunological response, cell-mediated and humoral immunity, primarily due to the human leukocyte antigen mismatches between donors and recipients (Bharat and Mohanakumar, 2007; Bharat et al., 2007; Bharat et al., 2008; Snyder et al., 2013). Th cells are known to play a critical role in the immune response (Sayegh and Carpenter, 2004; Nankivell and Alexander, 2010; Nazari et al., 2013). The differences of in stimulation could promote naïve CD4 T helper cell differentiation into Th1/Th17 or Th2/Tregs (Mosmann and Coffman,1989; Harrington et al., 2005). In allogeneic rejection, the mismatching of HLA tends to induce naïve CD4 Th to differentiate into Th1. These Th subsets have different characteristics and effects and secrete different cytokines (Mosmann et al., 1986). Th1/Th17 secretes IL-2, IL-17, TNF-α, IFN-γ and others, which can enhance allograft rejection. In contrast, Th2/Treg secretes IL-4, IL-5, IL-6, IL-10 and others, which can suppress cytokine secretion and function of Th1/Th17.

TCR stimulation can lead to the induction of several key signaling pathways for T-cell activation, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling. The NF-κB family of transcription factors contains five members: RelA (p65), RelB, c-Rel, p50 and p52, which can homo- and hetero-dimerize (Vallabhapurapu and Karin, 2009). In naïve T cells, NF-κB dimers are retained in the cytoplasm by the NF-κB inhibitor IκBα (Thome et al., 2010), a negative regulator of NF-κB. The phosphorylation and degradation of IκBα actives and allows NF-κB to translocate into the nucleus and drive gene transcription (Vallabhapurapu and Karin, 2009; Thome et al., 2010).

Abbreviations: CM, culture medium; CFA, complete Freund's adjuvant; FACS, flow cytometry; HLA, human leukocyte antigens; HIV, human immunodeficiency virus; H&E staining, hematoxylin-eosin staining; i.m., intramuscular injection; LNMCs, lymph node mononuclear cells; LDH, lactate dehydrogenase; MLC, mixed lymphocyte culture; MLR, mixed lymphocyte reaction; MHC, major histocompatibility complex; mAb, monoclonal antibody; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OVA, ovalbumin; PBS, phosphate-buffered saline; SMCs, splenic mononuclear cells; s.c., subcutaneous injection; Th, T helper cells; Treg, regulatory T-cells; Tk, trichosanthin; TCR, T-cell receptor

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NF-κB is one of the major transcription factors in T-cell activation that control allogenic immune responses *in vivo*.(Smith et al., 2006) NF-κB was considered to associate with allograft survival in allogeneic transplantation in many studies.(Finn et al., 2002; Yang et al., 2002) In allogenic pancreatic islet transplantation, rejection was delayed, with 40% of mice accepting islet allografts long-term when the mice were globally deficient in c-Rel (Yang et al., 2002).

Tregs, a functionally mature subset of $CD4^+$ T-cells that uniquely express CD25 and the nuclear *FOXP3* gene (Sakaguchi et al., 2006), are generated in the thymus during T-cell maturation or differentiate from naïve $CD4^+$ T-cells in the periphery. Tregs can provide immunological suppression during allogeneic rejection (Bilate and Lafaille, 2012; Josefowicz et al., 2012) by neutralizing killer T-cells (Issa and Wood, 2010) and suppressing immune responses. (Sakaguchi et al., 2001; Wood and Sakaguchi, 2003; Sakaguchi, 2011) Indeed, a number of studies have found that Treg-based therapies can extend allograft survival both in experimental animals(Tsang et al., 2009; Monteiro et al., 2010) and human patients (Sagoo et al., 2012; Schliesser et al., 2012). In addition, the phosphatidylinositol 3-kinase (PI3K) pathway is involved in various cell growth and survival signals in mammalian cells (Fruman and Cantley, 2002), especially in Tregs. PI3K signaling in Tregs is normally suppressed by the lipid phosphatase termed phosphatase and tensin homolog on chromosome 10 (PTEN) (Han et al., 2012). PTEN is important in Tregs science PI3K signals negatively regulate Foxp3 expression and Treg function (Crellin et al., 2007; Sauer et al., 2008).

Trichosanthin (Tk) is the principal active ingredient of the Chinese herb medicine isolated from *Trichosanthes kirilowii* Max (Wang et al., 1986; Yeung et al., 1987). Tk is a 247-amino-acid linear peptide, which has been used for abortion(Chan et al., 2003), cancer (Li et al., 2010) and AIDS human immunodeficiency syndrome.(Au et al., 2000) Tk has demonstrated immunological regulatory activity in inducing the type 2 immune response at physiological concentrations ($\leq$ 1 μg/ml)(Chou et al., 1994; Murata et al., 2001; Zhao et al., 2006; Zhou et al., 2007a,b). However, Tk therapy has several side effects, including myalgias, fevers, mild elevation in liver function tests and mild-moderate anaphylactic reactions (Byers et al., 1990; Byers et al., 1994). To overcome these side effects, a 40-amino-acid-long peptide was designed from the effective epitope of Tk based on a previous study and named Tk-PQ (Zhou et al., 2007a,b). We report here the cytotoxicity, immunological effects and mechanisms of Tk-PQ in allogeneic transplantation.

2. Materials and methods

2.1. Animals

Male C57BL/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice aged weeks were supplied by Shanghai laboratory animal center (Shanghai, China) and used in accordance with the regulations of the Scientific Investigation Board of the Shanghai Jiaotong University School of Medicine.

2.2. Reagents

The albumen trichosanthin injection (Tk, 1 mg/ml) was purchased from Shanghai Jinshan Pharmaceutical Co., Ltd (China). The Tk-PQ with above 95% purity was synthesized by GL Biochem (Shanghai) Ltd (China). The [^{3}H] thymidine ([^{3}H]-TdR) was purchased from Shanghai Institute of Applied Physics (China).

2.3. Mouse splenic or human peripheral blood mononuclear cell preparation

The spleen was removed from C57BL/6 or BALB/c mice and was prepared as a suspension in PBS through a 70-μm cell strainer (Falcon, BD, USA) in a sterile environment. Human peripheral blood was collected from volunteers. Mouse splenic mononuclear cells (SMCs) or the human peripheral blood mononuclear cells (hPBMCs) with a purity > 95% were isolated according to the protocol provided with the Lymphocyte Separation Medium (Dakewe biotech, China).

2.4. Mixed lymphocyte culture (MLC)

For the mouse MLC, the SMCs from C57BL/6 were used as responder cells (R), and 25 Gy-irradiated SMCs from BALB/c mice were used as stimulator cells (S). For human MLC, two sets of peripheral blood mononuclear cells (hPBMCs) from different humans without irradiation were used. Equal numbers (2×10^5) of R and S cells in 200 μl of RPMI-1640 medium (ThermoFisher, USA) supplemented with 10% fetal calf serum (ThermoFisher, USA), 100 U/ml penicillin and 100 μg/ml streptomycin were mixed and applied to round-bottomed 96-well microplates, followed by culturing at 37 °C and 5% CO_2 for 6 days. The addition of only one cell type served as a negative control. Sixteen hours before harvesting of the cultures, 0.5 μCi/well of [^{3}H]-TdR was added. The cells were then collected to determine the radioactivity with a liquid scintillation counter (MicroBeta TriLux, Turku, Finland). The suppressive effects of Tk or Tk-PQ on the mixed lymphocyte reaction (MLR) were expressed as the mean counts per minute (cpm) ± SD. The ± SD represent trebling to quintuple determination. Representative data from three independent experiments are shown.

2.5. OVA-specific lymphoproliferation

For OVA-specific lymphoproliferation, chicken OVA emulsified with CFA (Sigma Aldrich, USA) was injected s.c. at a concentration of 2 mg/ml into each hind footpad of the mice. After 9 days, the lymph node mononuclear cells (LNMCs) from OVA-primed mice were seeded at 2×10^5/well, followed by the addition of OVA (100 mg/ml) with/without Tk or Tk-PQ and incubation for 5 days. The negative control contained the addition of LNMCs only. The intensity of lymphoproliferation was determined by [^{3}H]-TdR.

2.6. Assessment of Tk and Tk-PQ cytotoxicity

To assess the cytotoxicity of Tk or Tk-PQ on SMCs, the MLR of SMCs was set up as described. After incubation for 3 days, the cytotoxicity of Tk and Tk-PQ were analyzed using the LDH cytotoxicity assay kit (Promega, USA). The methods followed the protocol supplied with the kit. For the *in vivo* analyses, fifteen 8-week-old C57BL/6 mice were randomly assigned to three groups (5 mice/group) for injection i.m. of 50 μl of Tk (0.1 mg/ml) or Tk-PQ (1 mg/ml) every day for 2 weeks. The control group was injected with 50 μl phosphate-buffered saline (PBS). The mouse weights were determined once on two days.

2.7. Adoptive experiment of culture supernatant from MLR

The SMC MLR was performed as described with 1 μg/ml Tk or 50 μg/ml of Tk-PQ. The group without Tk and Tk-PQ was used as a control. After incubation for 5 days, the culture medium (CM) was collected and re-added to the new SMC MLR. The new SMCs were incubated for 3 days. The intensity of lymphoproliferation was determined by [^{3}H]-TdR.

2.8. Antibody blocking experiment

After performing the SMC MLR as described, Tk-PQ was added at 50 μg/ml. Additionally, anti-IL-10 or anti-TGF-β1 (R&D Systems, USA) or anti-IL-10 plus anti-TGF-β1 monoclonal antibody (mAb) was added at 20 μg/ml. The SMCs without Tk-PQ and antibody was used as a negative control. The isotype group was supplemented with mouse IgG. After incubation for 5 days, the intensity of lymphoproliferation was determined by [^{3}H]-TdR.