Original Article

Pentapeptide PLNPK ameliorates adjuvant arthritis and inhibits T cell activation by suppressing Lck and PI3K activities

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Abstract: The pentapeptide PLNPK could inhibit the transformation and proliferation of mouse T lymphocytes in mice. Rheumatoid arthritis (RA) is essentially a kind of T cell-mediated arthritis. Therefore, we assessed the therapeutic ability of PLNPK on adjuvant arthritis (AA) rats, and examined its effects on signals of CD4+ T cell activation. Isolated CD4+ T cells were stained with CFSE, and stimulated by anti-CD3 or anti-CD3 and anti-CD28 monoclonal antibodies. The suppression effects of PLNPK on T cell proliferation and CD25 expression were detected by flow cytometry. AA rats received PLNPK or saline intraperitoneal injection once a day for 20 days. Effects on disease progression were assessed by measurement of paw swelling. Inflammation and joint destruction were examined by histology, T cell infiltration and activation in joint were detected by immunohistochemistry stain. The suppression effects of PLNPK on Lck and PI3K ability in CD4+ T cells and in AA rats were assessed by immunoprecipitation and kinase assay kits. PLNPK inhibited the CD4+ T cell proliferation and altered their CD25 expression when they received CD3 mAb or CD3/CD28 mAbs stimulation. In AA rats, PLNPK inhibited T cell infiltration and activation in joint. In addition, PLNPK inhibited Lck activity in CD3 mAb stimulated CD4+ T cells as well as PI3K activity in CD3/CD28 mAbs stimulated CD4+ T cells in vivo. PLNPK can also reduce the Lck and PI3K activities in AA rat splenocytes. PLNPK exerts its immunosuppressive effects and ameliorates adjuvant arthritis by inhibiting the Lck and PI3K activities in T cells.

Keywords: Rheumatoid arthritis, immunosuppressive agents, flow cytometry, immunohistochemistry, enzymes

Introduction

As the largest immune system organ in the body, the spleen contains many immunocytes and cytokines, they play important roles in immune regulation. A large number of peptides with immunological activities have been extracted from spleen, and some of them are potential to be medicine. The pentapeptide PLNPK (Pro-Leu-Asn-Pro-Lys) is one of them. Our previous studies showed that PLNPK could inhibit ConA-induced T proliferation, and could inhibit immunoglobulin production by B cells in mice, suggesting that PLNPK is an immunosuppressive oligopeptide [1, 2]. The latest studies discovered the therapeutic effects of PLNPK on autoimmune diseases in animal models, such as anti-glomerular basement membrane (GBM) nephritis [1] and systemic lupus erythematosus (SLE) [3], and PLNPK also prolonged the survival time of skin allograft and cardiac allograft [2]. In addition, we found the common mechanism by which PLNPK plays its therapeutic roles is to inhibit T cell activation [2, 3].

CD4+ T cells play a central role in the immune response. The mechanisms governing CD4+ T cell activation require fine-tuning, because hyperactivation can lead to immunoproliferative disorders and autoimmunity. We firstly studied the impacts of PLNPK on CD4+ T cell activation. Peripheral T cells are activated following TCR/CD3 triggering in the presence of costimulatory signals. The earliest recognizable event after...
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T cell receptor (TCR) engagement by antigen is the activation of protein tyrosine kinases (PTK) of Src family [4, 5]. The Src kinase family member p56lck (Lck) is the major PTK during TCR triggering. Lck is mainly responsible for the activation of phospholipase Cγ (PLCγ). PLCγ initiates the subsequent Ca²⁺/calmodulin/calcineurin/NFAT pathway and PKC/NF-κB pathway [5]. In addition, Lck is involved in ERK/MAPK signal activation [6-8]. Therefore Lck is critical for TCR triggered T-cell activation. In this study, we observed the inhibitory effect of PLNPK on Lck in T cells.

In addition to TCR/CD3 signal, complete activation of T cells also needs the second signals provided by pairs of costimulatory molecules, the most important one of which is B7/CD28. CD28 binding to its ligand B7 results in tyrosine phosphorylation of Y173MNMT motif. The phosphorylated YMNM motif subsequently interacts with the SH2 domain within phosphoinositide 3-kinases (PI3K) regulatory subunit p85, activating the p110 catalytic subunit [9]. The p110 generates PIP3 that recruits a variety of proteins to plasma membrane, among which is phosphatidylinositol-dependent kinase-1 (PDK-1). PDK-1 activates AKT, and AKT can significantly promote the T cell activation and proliferation, cytokine secretion, inhibits Fas-mediated T cell apoptosis [10-12]. We also studied the effect of PLNPK on PI3K activity in T cell.

Rheumatoid arthritis (RA) is essentially a kind of T cell-mediated arthritis, RA disease originated in T cell activation [13]. A lot of self-reactive CD4⁺ T cells were detected in joints of RA patients, these abnormal T cells interacts with other pathogenic cells through direct contact or secretion of causative agents [14-16]. We established rat adjuvant arthritis (AA) model, observed the effect of PLNPK treatment on autoimmune diseases AA, and studied the effect of PLNPK on T cell activation in vivo.

Materials and methods

Drug and reagents

PLNPK was obtained from Shenzhen Kangzhe Pharmaceutical Co., Ltd. Anti-human CD3 mAb, anti-human CD28 mAb, anti-human CD4-PE antibody and anti-human CD25-APC antibody were purchased from BD-PharMingen company. Carboxyfluorescein diacetate succinimidyl ester (CFSE) and Freund’s complete adjuvant were purchased from Sigma-Aldrich Corporation. Anti-Lck mAb and anti-P85 mAb was purchased from Upstate Company. PTK kinase assay kit was purchased from Sigma-Aldrich Corporation. PI3K kinase assay kit was purchased from Echelon Biosciences company, anti-rat CD3 mAb and anti-rat CD25 mAb were purchased from Serotec Company.

Animals

Male Wistar rats (SPF grade, 180 ± 20 g), were provided by Experimental Animal Center of Military Medical Sciences. They were housed in an environment with a controlled 12:12 light/dark cycle and constant temperature of 21-25°C with a humidity of 55% ± 5%. All procedures carried out according to NIH’s SOP Practice.

Ethics statement

Venous blood samples were collected from health volunteers with informed consent and with the approval of local ethics committee and Health Bureau. Animal experiment in this study was approved by Ethics Committee of Tianjin Medical University.

Isolation of primary peripheral blood CD4⁺ T cells

The peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient (1.077 g/ml) centrifugation at 2000 rpm for 20 minutes. After washing 3 times, CD4⁺ T cells were negatively selected by an antibody cocktail according to the manufacture’s instructions (Miltenyi Company). A Cell purity of above 90% was achieved by flow cytometry detection.

CD4⁺ T lymphocyte proliferation

CD4⁺ T cells were adjusted to 1×10⁶/ml, incubating with carboxyfluorescein succinimidyl ester (CFSE, 5 mmol/ml) in 37°C for 15 minutes for CFSE labeling. Finally, the reaction was stopped by ice bath for 5 minutes. After washing for 3 times, CD4⁺ T cells were resuspended in RPMI-1640/10% FBS and plated in 96-well plate at 4×10⁵/well along with titrating doses of PLNPK at a final concentration of 1.6 mg/ml.
0.8 mg/ml, 0.4 mg/ml, 0.2 mg/ml or media control. Except for unstimulated control group, cells were stimulated with 2.5 μg/ml CD3 mAb alone or CD3 mAb (2.5 μg/ml) and CD28 mAb (1 μg/ml) both. The plate was placed in 37°C, 5% CO₂ incubator for 48 hours. Data acquisition was processed by CELLQuest software with flow cytometry (BD-PharMingen Corporation), and the proliferation index was obtained by Modfit software.

**CD25 expression in T lymphocytes**

In this study, 2×10⁶ purified human CD4⁺ T cells were plated in 24-well plate with the same final concentrations of PLNPK as above. Cells also received the same stimulation as above, and the unstimulated control wells received no stimulation. The plate was placed in 37°C, 5% CO₂ incubator for 48 hours. After incubation, the cells were harvested and stained with anti-human CD4-PE and CD25-APC antibodies for evaluation of T cell activation. Data acquisition was processed by CELLQuest software with flow cytometry (BD-PharMingen Corporation).

**AA model and drug treatment**

AA model was established by intradermal injection of 0.1 ml FCA in the right hind paw of each rat. After 7 days, the rats were randomly divided into four groups: different doses of PLNPK (200 μg/kg/d, 100 μg/kg/d), model control group and healthy control group, there ten animals in each group. The 2 PLNPK groups received 1 ml i.p. injection of 200 μg/kg/d and 100 μg/kg/d PLNPK respectively once a day for 20 days, at the same time, the two control group were treated with 1 ml saline.

**Histological examination**

After the last administration, all of the AA rats were sacrificed, and each right ankle was removed, washed with saline and fixed in 10% neutral formalin. After 15% EDTA (2Na⁺) decalcification, conventional dehydration and paraffin embedding, sliced, the joints were stained by haematoxylin-eosin (HE) for light microscope examination. In addition, some paraffin sections were microwave repaired, and then were incubated with anti-CD3 mAb or anti-CD25 mAb working solution at 4°C overnight. On the next day, HRP labeled goat anti-mouse IgG and DAB were used to reveal the reaction. Positive cells were with brown-yellow or yellow granular material deposits to the cytoplasm.

**Lck activity detection**

For in vitro study, 2×10⁶ purified human CD4⁺ T cells were added in 24-well plate with the same PLNPK treatment as above. Cells were stimulated with 2.5 μg/ml CD3 mAb except for that in unstimulated control wells. The plate was placed in 37°C, 5% CO₂ incubator for 48 hours. Total protein was extracted by RIPA lysis buffer, and was quantified to a final concentration of 5 μg/μl. Lck samples were prepared by immunoprecipitation as follows: 1 mg total protein was incubated with 3 μl Lck antibody for one hour at 4°C, and then incubated with 60 μl 50% Protein A bead at 4°C for one hour. The solution was centrifuged at 14,000 g for 5 seconds and the supernatant was discarded. After washing, the samples could be used for the evaluation of Lck kinase activity. PTK kinase assay kit was used to detect the Lck activity.

In order to observe the effect of PLNPK on Lck activity in vivo, total proteins were extracted from spleen tissue of AA rats in each group. Lck samples were prepared by immunoprecipitation, and PTK kinase assay kit was used to detect the Lck kinase activity.

**PI3K activity detection**

2×10⁶ purified human CD4⁺ T cells were plated in 24 well plates with the same PLNPK treatment as above. Cells were stimulated with 2.5 μg/ml CD3 mAb and 1 μg/ml CD28 mAb in stimulation wells, and unstimulated control received no stimulation. The plate was placed in 37°C, 5% CO₂ incubator for 48 hours. After that, the samples of p85 subunit were prepared by immunoprecipitation as above, and then, the activity was examined with a PI3K kinase assay kit. The PI3K activity in splenocytes of AA rats was also detected.

**Statistical analyses**

Data were expressed as group means ± standard deviation and analyzed with one-way analysis of variance (ANOVA). *P*<0.05 was considered to be statistically significant.

**Results**

**PLNPK inhibited the proliferation of CD4⁺ T cells**

In order to observe the effect of PLNPK on CD4⁺ T cells proliferation, we used CD3 mAb and
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Figure 1. Effect of PLNPK on McAb induced CD4+ T cells proliferation. Human CD4+ T cells were labeled with CFSE and plated in 96-well plate with titrating doses of PLNPK, and were simulated with CD3 McAb (A, B) or CD3/CD28 McAb (A, C). After cultured for 48 hrs, cell proliferation was detected by flow cytometry. Data are expressed as mean ± SD, *: versus stimulated control group, P<0.05, N=3.
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Figure 2. Effect of PLNPK on CD4⁺ T cells CD25 expression induced by antibodies. Human CD4⁺ T cells were plated in 96-well plate with titrating doses of PLNPK, and were simulated with CD3 McAb (A, B) or CD3/CD28 McAb (A, C). After cultured for 48 hrs, cells were stained with anti-human CD4-PE and CD25-APC antibodies and were detected by flow cytometry method. Data are expressed as mean ± SD, *: versus stimulated control group, P<0.05, N=5.
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Figure 3. Effect of PLNPK on the ankle joints’ pathology and T cell infiltration of AA rats. AA model was induced by intradermal injection of FCA in right hind footpads of rats, PLNPK were administrated continuously for 20 days from the seventh day after immunization. The histological change of ankle joint were examined under light microscope after HE stain and immunological histochemistry stain. A: Saline control (×100) synoviocytes accrementition on cartilage and pannus. Mononuclear cells infiltrate severely. B: PLNPK (200 μg/kg/d) (×100) group, cartilage destruction and synoviocytes accrementition were relieved. Mononuclear cells infiltration was lessen. C: PLNPK (100 μg/kg/d) (×100) group, cartilage destruction and synoviocytes accrementition were relieved. Mononuclear cells infiltration was lessen. D: Saline control (×100), CD3+ T cells infiltrated to synovial tissue severely. E: PLNPK (200 μg/kg/d) (×100) group, CD3+ T cells infiltration was lessen. F: PLNPK (100 μg/kg/d) (×100) group, CD3+ T cells infiltration was lessen. G: Saline control (×100), CD25+ T cells infiltrated to synovial tissue severely. H: PLNPK (200 μg/kg/d) (×100) group, CD25+ T cells infiltration was lessen. I: PLNPK (100 μg/kg/d) (×100) group, CD25+ T cells infiltration was lessen.

CD3/CD28 mAbs to stimulate CD4+ T cells, PLNPK concentrations were 1.6 mg/ml, 0.8 mg/ml, 0.4 mg/ml, 0.2 mg/ml. The results showed that the proliferation kinetics, as shown by CFSE dilution profiles, was strikingly different between stimulated control and unstimulated control, suggesting that CD3 mAb (2.5 μg/ml) and CD3/CD28 mAbs (2.5 μg/ml/1 μg/ml) can induce CD4+ T cells proliferation. As expected, anti-CD3 plus anti-CD28 costimulation activated more T cells. All the doses of PLNPK inhibited both CD3 mAb-induced and CD3/CD28 mAb-induced CD4+ T cell proliferation in a dose dependent manner, there was significant difference versus the stimulated control group, \( P<0.05 \) (Figure 1).

PLNPK reduced the activation of CD4+ T cells

The CD25 expression is a critical step in T cell activation. When the T cells are activated, they produce the α chain of IL-2 receptor (CD25), which contributes to IL-2 binding. CD25 is also an important marker of activated T cells. So we detected CD25 expression to evaluate the effect of PLNPK on CD4+ T cell activation. The positive rates of CD25 in stimulated control groups is much higher (47.1 ± 6.6% for anti-CD3 stimulated control and 51.9 ± 7.1% for anti-CD3/CD28 stimulated control) than that of unstimulated group (less than 10%), PLNPK (1.6, 0.8, 0.4 and 0.2 mg/ml), significantly inhibited the expression of CD25 both in
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CD3 stimulated groups and in CD3/CD28 stimulated groups in a dose dependent manner, \( P<0.05 \) versus stimulated control (Figure 2).

**PLNPK reduced AA pathological damage and inhibited joint T lymphocytes infiltration**

AA and RA have similar pathological changes, including synovial hyperplasia, mononuclear cell infiltration, cartilage and bone destruction. There are excessive growth of various cells in the synovial tissue, including T cells and polyclonal B cell, macrophage-like synovial cells, monocytes, and so on, which produce monokines and chemokines to stimulate synovial cell proliferation and polymorphonuclear leukocytes driven into the joints, resulting in proliferation of synovial tissue, pannus formation, cartilage and bone destruction [17, 18]. In our study, the right ankle joints of model control AA rats showed synovial cell proliferation, synovial thickening, pannus formation, and a large number of inflammatory cell infiltration, cartilage damage was serious; AA rats in PLNPK treatment groups exhibited ameliorated joint pathological changes of different degrees, for instance, mild synovial hyperplasia, cartilage erosion were worm-like changes, infiltration of inflammatory cells was decreased (Figure 3A-C). T cells are the major AA synovial infiltrating cell subsets, it even can induce germinal centers formation in synovial structure, inflammatory cytokines excretion, synovial tissue proliferation, B cell activation and Ig secretion, eventually leading to synovial membrane injury [19]. CD25 is a surface marker of T cell activation, and CD25^+ synovial T cells significantly increased in RA patients. Our results revealed that a large number of CD3^+ T cells, CD25^+ T cells infiltrated in synovial tissue in the model control group as well as in PLNPK treatment groups, there is no significantly deferent among these groups.

**PLNPK inhibited Lck activity**

The Src PTK family Lck is essential for T cell development and T cell receptor (TCR) signalling. After activated by anti-CD3 antibody, Lck activity is much higher than that of unstimulated cells. PLNPK (1.6, 0.8, 0.4 and 0.2 mg/ml) reduced the Lck activity of CD3 mAb stimulated human CD4^+ T cells, and there was significant difference versus control group, \( P<0.05 \) (Figure 4A).

During AA pathogenesis, Hsp65 protein can activate specific T cell clones, causing immune response to the synovial membrane. It is reported that Lck inhibitors can inhibit the abnormal activation of T cells in AA animals, and reduce the early symptoms [20]. Our results suggested that Lck activity in AA rat splenocytes was higher that in normal rats, PLNPK in 200 μg/kg/d and 100 μg/kg/d dose, was able to significantly inhibit Lck activity compared with control group, \( P<0.05 \) (Figure 4B).

**PLNPK inhibited PI3K activity**

P13K aggregation and activation play a key role in CD28 signal pathway. When CD28 combined
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with and its ligand, Tyr173 in cytoplasmic domain of CD28 is phosphorylated ensue on P13K activation and ultimately, induce AP-1 and NF-AT transcription [21]. In order to observe the effect of PLNPK on P13K, CD3/CD28 mAb were used to stimulate CD4+ T cell activation, PI3K activity was detected by ELISA. The results discovered that CD3/CD28 mAb (2.5 μg/ml/1 μg/ml) increased CD4+ T cells PI3K activity, and all the dosages of PLNPK (1.6, 0.8, 0.4 and 0.2 mg/ml) inhibited CD3/CD28 mAbs induced human CD4+ T cells PI3K activity, there was significant difference versus control group, P<0.05 (Figure 5A).

In AA animals, PI3K pathway is involved in the pathologic processes. studies have shown that application of specific PI3K inhibitor wortman- nin or LY294002, can inhibit T cell secretion of TNF and IL-10 in AA, and inhibit the activation of macrophage induced by T cells directly [22, 23]. Our results show that, PLNPK in 200 μg/kg/d and 100 μg/kg/d dose, was able to significantly inhibit AA rat splenocytes PI3K activity, compared with control group, P<0.05. Inhibition rates were 61.74% and 59.44% respectively (Figure 5B).

Discussion

Activation of T cells is a central process in immune response, so inhibiting T cell activation is an important target for the development of immunosuppressive agents. There has been found that certain oligopeptides and polypep-

tides can simulate or block specific protein-protein interactions of T cells. The mechanisms of those inhibitory peptides include two aspects: inhibition of the first signal, and inhibition of the costimulatory signal of T cell activation. The HLA-DQα1 peptide is derived from human MHC II molecules α chain, it can interfere with the interaction between TCR and MHC and inhibit cytotoxic T cells generation [24-26]. Another example is CD80-CAP1 (MQPPGC), it can block combination of CD80/86 and CD28/152, and is effective to prevent and suppress autoimmune disease symptoms of collagen-induced arthritis in mice (CIA), inflammatory bowel disease (IBD), experimental allergic encephalomyelitis (EAE) and trinitrobenzene sulfonic acid (TNBS) colitis [26-30].

The spleen contain a variety of biologically active substances, PLNPK is a kind of immunosuppressive peptide screened from spleen. This study showed that, PLNPK could significantly inhibit CD3 mAb and CD3/CD28 mAbs induced CD4+ T cells proliferation and activation. CD4+ T cells play an important role in assisting B cells to produce immunoglobulin, supporting NK cell and CD8+ Tc cells activation and secretion. The effect of PLNPK on CD4+ T cells suggests that it is a potential immunosuppressive agent.

In vitro, CD3 mAb triggered the first signal transduction pathway of T cell activation. At first TCR/CD3/CD45/CD4 rapidly aggregate, then CD45 exerts protein tyrosine phospha-
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tase activity resulting Src PTK phosphorylation,
followed by activation of ZAP-70, LAT [4, 31].
The ability of PLNPK to inhibit Lck protein tyrosine kinase activity is likely to lead to inhibit
the first signal transduction pathway of T cell activation. The expression of CD25 can be
used as a sign of T cell activation. Inhibition of Lck activity may be the mechanism of PLNPK
treatment reduced CD3 mAb stimulated CD25 expression.

CD28 mediates a costimulatory signal that cooperates with TCR/CD3 activation to promote T cell viability, clonal Expansion and cytokine production. Ligation of CD28 by Abs or
its natural ligands B7-1 and B7-2 results in tyrosine phosphorylation at Y173MNM motif
within its cytoplasmic tail. The phosphorylated YMNM motif subsequently interacts with the
Src homology 2 domain within the p85 regulatory subunit of PI3K, activating the p110 catalytic subunit. Activated PI3K generates intracellular PI [3-4] P2 and PI [3-5] P3, thereby
recruiting numerous proteins to plasma membrane by pleckstrin homology (PH) domains including AKT and PDK-1. Akt, upon subsequent phosphorylation at Thr-308 and Ser-473 by
PDK-1, becomes activated and plays a critical role in cell survival and cell cycle regulation through a wide variety of downstream molecules [9, 10, 32, 33]. PLNPK significantly inhibits CD3/CD28 mAb induced PI3K activity, indicating another mechanism of PLNPK function.

Rheumatoid arthritis is essentially a T cell-mediated arthritis. Abnormal T-cell repository is the internal factor of RA pathogenesis. There are a lot of self-reactive CD4+ T cells control the occurrence and development of RA through direct contact or secretion [34]. Experiments confirmed that, direct specific T cell clones injection into normal animals can lead to destructive arthritis [35]. In this study, rat AA model was established to observe the immunosuppressive activity of PLNPK in vivo. During AA pathogenesis, Rheumatoid-associated antigens were presented through HLA, and combined with the TCR/CD3 of T cells. In the presence of costimulatory signals, the T cells were abnormally activated. Those T cells were recruited into the joints, resulting in the occurrence of joints inflammation [36, 37]. PLNPK can inhibit Lck and PI3K activity of T cells in AA rat, naturally inhibits the T cell downstream signal transduction. Thereby, PLNPK can reduce the T cell-mediated inflammatory response, improve the ankle pathological damage.

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Disclosure of conflict of interest
None.

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