Cloning of interleukin-15 gene of Tibetan pig and adjuvant effect of its recombinant plasmids packed with PEG and PEI modified chitosan nanoparticles on immunity of mice to FMD vaccination

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Abstract

IL-15 cDNA of Tibetan pig was firstly cloned from its activated lymphocytes, and then was sub-cloned into VR1020 to construct recombinant VRTIL-15 plasmid to study the in vitro and in vivo biological effects on animal. The VRTIL-15 was entrapped with chitosan modified with PEG-PEI (CS-PEG-PEI) to transfet HEK293 cells for the preliminary study of its expression in eukaryotic cells. The total RNA of HEK293 cell was isolated in 48h, and the successful expression of IL-15 was detected by RT-PCR and the supernatant of HEK293 cells was found to stimulate significant proliferation of lymphoblasts of pig. Subsequently, VRTIL-15 packed with CS-PEG-PEI was utilized to intramuscularly inoculate Kunming female mice at the age of 21 days. Their bloods were collected before and after inoculation on 1, 2, 3, 4 and 5 weeks to detect the changes of innate and adaptive immunity of animals. The results were found that Th and Tc, specific antibody to FMD, IgG, IgG1, IgG2a content markedly increased in the blood of treated mice compared with the control group (P<0.05). The mRNA expression of TLR1, TLR4, TLR6, TLR9, TGFB- β , IL-2, IL-4, IL-6 and IL-23 were significantly higher in the treated group than those of the control (P<0.05). These results indicate that the VRTIL-15 wrapped with CS-PEG-PEI can significantly improve the innate, humoral and cellular adaptive immunity of animal, which could inspire the development of effective immune adjuvant to improve the comprehensive immune protection of animals against FMD.

Keywords: Chitosan and its derivatives, nanoparticle package and delivery, gene expression, pig interleukin-15, mice, immunity
1. Introduction

The Tibetan pig is the unique indigenous pig breed in China, and has evolved for nearly nine thousands of years through natural selection in the Qinghai-Tibetan Plateau with an average altitude of more than 4200 m above sea level. It become adapted to marginal feeding and harsh environment on the plateau and is well known for strong resistance against various diseases and hypoxia environment\(^1\). But up to now little is known about the molecular mechanisms for these unique performances, especially for its special immunity against diseases.

The interleukin-15 (IL-15) is an important cytokine in immune regulation of animal immune system, and has a key role in the proliferation, survival and activation of CD8+ T, natural killer (NK) and other immune cells\(^2,3,7\). Although its function is similar to IL-2, IL-15 utilizes different mechanisms of signaling with IL-15 receptors to affect multitudinous target cells. Nowadays there is no report about IL-15 of Tibetan pig and far less than its role in the immune responses to viral vaccination of animal. To further explore and clarify the immunogenetic characteristics of Tibetan pig, here we conducted the present experiment to clone IL-15 gene of Tibetan pig and analyze its potential as an immunoadjuvant to boost the immune responses to Foot and Mouth Disease (FMD) vaccine which is still in urgent need of safe and effective adjuvant.

As a non-toxic, biocompatible and biodegradable polysaccharide, chitosan (CS) has attracted great attention in recent years, and a number of applications in drug delivery have been found due to its favorable biological properties\(^4,5,16,18\). In order to improve the efficiency of \textit{in vivo} gene transfection in naimal, different chitosan derivatives were prepared and employed to pack the recombinant expression plasmid for IL-15 gene in animal vaccination experiment.

2. Materials and methods

2.1. TPIL-15 gene cloning and sequencing

Total RNA was extracted from the collected leukocytes of a Tibetan pig which was stimulated by LPS for 12 hours. RT-PCR is used for TPIL-15 gene cloning into pMD\(^\oplus\)19-Tvector (Takara), and then transformed into DH5\(\alpha\) competent cells. Primers were designed by Primer 5.0 with \textit{BamHI} and \textit{BglII} sites according to conserved ORF sequence of IL-15 gene of Duroc pig, Landrace pig and other mammalians from NCBI/GenBank. PCR primers for the IL-15 sequences are: TPIL-15-F: 5’ATGTGTTTGAGAAGTACTTG3’, TPIL-15-R: 5’GTTCATCAACCCTTCTTGA3’. Plasmids were assessed for conformity by PCR and digested by \textit{BamHI} and \textit{BglII}. Subsequently, the pMD\(^\oplus\)19-T/IL-15plasmids were sent to BGI biological company for sequencing. TPIL-15 sequence was aligned with domestic pigs and other mammalians by NCBI/Blast. Plasmid VR1020 is a eukaryotic expression vector (Vical Company of America). We cloned the TPIL-15 into VR1020 to construct VTPIL-15 to get secreted protein for \textit{in vitro} and \textit{in vivo} bioactivity test using the same fragment and methods as above mentioned.

2.2. Large-scale preparation of recombinant VTPIL-15, chitosan nanoparticles and characterization

A monoclonal cell line of recombinant DH5\(\alpha\) E. coli, containing VTPIL-15 or VR1020 plasmid, was inoculated in LB broth with kanamycin (100 mg/ml) at 37\(\degree\)C, 200rpm overnight. Plasmid was extracted following the spermine precipitation method described as Jason then the plasmid was resuspended in sterile water and stored at -20\(\degree\)C until use.

The VTPIL-15 plasmids were entrapped with chitosan (CS, provided by Chengdu Organic Chemistry Institute of Chinese Academy of Science, China, MW: 50 kD and its deacetyl degree is over 95 percent) and its derivatives (CS-PEG–PEI and CS-PEG-LAC, prepared in our lab as reported previously\(^{19,20}\)) by ionotropic gelation method\(^15,16\). The zeta potential and average diameter of the nanoparticles were characterized by Zeta-sizer 3000 HS/IHPL (Malvern Instruments Ltd., Malvern, UK).

2.3. Gene transfection and bioactivity analysis

HEK293 cells were seeded in 12-well plates (1.0 \(\times\) 10\(^5\) cells/well) respectively. The cells were incubated in Dulbecco’s modified Eagle medium (DMEM, Invitrogen Corporation.). The nanoparticles enwrapped with CS, CS-PEG–PEI and CS-PEG-LAC containing 3 \(\mu\)g of DNA were added into the wells, respectively, then they were incubated at 37\(\degree\)C in a 5% carbon dioxide humidified atmosphere.

The bioactivity of the TPIL-15 protein was measured by its ability to provoke the proliferation and cell viability of pig lymphoblast stimulated with Concanavalin A (Con A) through CKKS colorimetry. Lymphocyte Separation Medium (LSM, ficoll 400) was used to separate pig peripheral blood mononuclear immune cells for \textit{in vitro} bioactivity test.
2.4. Animal vaccination

Forty 4-week-old female Kunming mice (purchased from the Animal Center of West China Center of Medical Sciences, Sichuan University) were randomly assigned to four groups (A1, A2, A3, C), 10 mice in each group. All mice were muscually injected with 0.2 ml inactivated FMD vaccine (OZK93 + OS99 strain from Lanzhou Veterinary Institute, China), 100μg the plasmids per mouse in the left and right quadriceps on day 0. Mice in group A1 were injected with VTPIL-15/CS, A2 with VTPIL-15/CS-PEI-PEG, A3 with VTPIL-15/CS-PEI-LAC and group C were injected with VR1020/CS as a negative control. Peripheral blood samples were collected from the tail vein of mice on days 0, 7, 14, 21, 28 and 35 after immunization.

2.5 Bioactivity assay of TPI15 in vivo

2.5.1 Changes of immune cells in peripheral blood

Peripheral blood samples were used EDTA for anticoagulation, double diluted with normal saline, then analyzed by blood testing instrument: MIND-RAY BC-3000 intelligent automatic blood cell analyzer. Blood test include red blood cells, white blood cells, hemoglobin and platelet count and so on.

2.5.2 Assay of CD3, CD4 and CD8 positive T cells by FCM

Monoclonal antibody for FCM including Anti-Mouse CD3e FITC(0.5ug/Test), Anti-Mouse CD8a PE (0.25ug/Test), and Anti-Mouse CD4 PerCP-Cy5.5(0.25ug/Test) for each Test contain 50μl peripheral blood (monoclonal antibody were purchased from eBioscience Company, San Diego, USA,). FACS Aria BD was used for FCM analysis(BD Biosciences, USA).

2.5.3 Measurement of antibody, IgG, IgG1 and IgG2a

Antibody to FMD, mouse IgG, IgG1, IgG2a measurement ELISA kits were respectively purchased from Keqian Company of Wuhan, China and Bethyl Laboratory, Inc., America. The sandwich ELISA was conducted according to the manufacturer’s protocols.

2.5.4 Analysis of immune gene mRNA expression

According to reports in GenBank, 10 pairs of specific primers for mouse β-actin, TRL1, TLR4, TLR6, TLR9, TGF, IL-2, IL-4, IL-6 and IL-23 cDNA sequence were designed and synthesized (Table 1.).

<table>
<thead>
<tr>
<th>Genes</th>
<th>primers (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>β-actin-F</td>
<td>TACGCCAACACGGGTGCTGTC</td>
</tr>
<tr>
<td>β-actin-R</td>
<td>GTACTCCGTGCTGATCCACAT</td>
</tr>
<tr>
<td>TLR-1-F</td>
<td>GGACCTACCTTGCAAACAA</td>
</tr>
<tr>
<td>TLR-1-R</td>
<td>GGTGGCCACACATCACCTTT</td>
</tr>
<tr>
<td>TLR4-F</td>
<td>ACCCTGCGTCTTACACGC</td>
</tr>
<tr>
<td>TLR4-R</td>
<td>CTGCCAGAGACAATTGCAGAA</td>
</tr>
<tr>
<td>TLR-6-F</td>
<td>CCAAGAAACAAAAGCCCTGAG</td>
</tr>
<tr>
<td>TLR-6-R</td>
<td>TGGTTTGCAACCCGATTTGCTT</td>
</tr>
<tr>
<td>TLR9-F</td>
<td>ACTGAGCACCCTGCTTCTA</td>
</tr>
<tr>
<td>TLR9-R</td>
<td>AGATTAGTCAGCGCAGGAA</td>
</tr>
<tr>
<td>TGF-β-F</td>
<td>GGGAGTAGACAAGGATACACAC</td>
</tr>
<tr>
<td>TGF-β-R</td>
<td>ACACACAGCCTACTT</td>
</tr>
<tr>
<td>IL-2-F</td>
<td>AGCTCGGAGGAGGATGTCTA</td>
</tr>
<tr>
<td>IL-2-R</td>
<td>TGGTTACGATCCCCACATTTC</td>
</tr>
<tr>
<td>IL-4-F</td>
<td>GCCATATCCACGGATCGGAC</td>
</tr>
<tr>
<td>IL-4-R</td>
<td>GGTGTTCTCTGCTGATGAGA</td>
</tr>
<tr>
<td>IL-6-F</td>
<td>TCTTGGGACTGATGCTGAGA</td>
</tr>
<tr>
<td>IL-6-R</td>
<td>AGCCCTCGACTTGTGAAAGTAGTAT</td>
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</tbody>
</table>
Quantitative real-time PCR (IQ5 Real Time PCR cycler, Bio-Rad) was performed to analyze the gene expression of TRL1, TLR4, TLR6, TLR9, TGF-β, IL-2, IL-4, IL-6 and IL-23 and one reference genes, β-acting. In brief, 1 µL of cDNA, primers, 5'-6-carboxyfluorescein (FAM) were added to a total of 25 µL in 1× TianGen™ QPCR Mix (TianGen Peking, China). The relative expression of cytokine mRNA was calculated relative to the mean expression of the cytokines or TLRs mRNA in the mice using the geometric means method and the formula: relative level = $2^{-\Delta\Delta C_{T}}$. Significant differences between the experimental groups were calculated for each gene using a Mann–Whitney rank sum test on the relative cytokines/TLRs levels.

2.6 Statistical analysis

Data from all groups were presented as means ± SD, and statistical significance of the data was analyzed using Student’s t-test. Differences between experimental groups were considered to be significant when P-value was less than 0.05.

3. Results

3.1 TPIL-15 gene cloning and sequencing

We successfully cloned TPIL-15 gene from the activated immune cells in blood. PCR products amplified consistent with the expected size TPIL-15 gene-specific fragments. TPIL-15 gene cDNA is 474 bp of encoding sequence, and 158 amino acids. TPIL-15 gene alignment with the NCBI pig IL-15 cDNA, have more than 99% homology, and the cDNA sequence of TPIL-15 was submitted to GenBank, Sequence ID: gb|KF246518.1|

3.2 Size and potential of plasmids nanoparticles

The cationic nanoparticles had been considered as excellent candidates for gene transfection and expression in cells. Under the transmission electron microscope, most of the nanoparticles were spherical and the average granule diameter was 210 nm (range from 181 to 276 nm) analyzed by Zetasizer 3000 HS/IHPL, the diversity of chitosan nanoparticles was 0.167, zeta potential was 40.57 mV revealed in Table 2. The nanoparticles package of recombinant plasmids

<table>
<thead>
<tr>
<th>Sample</th>
<th>$T^{(\circ C)}$</th>
<th>w/w</th>
<th>Average</th>
<th>SD(±)</th>
<th>Average</th>
<th>SD(±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS/DNA</td>
<td>25</td>
<td>30: 1</td>
<td>222.2</td>
<td>5.15</td>
<td>+40.57</td>
<td>1.6</td>
</tr>
<tr>
<td>CS-PEG-PEI/DNA</td>
<td>25</td>
<td>10: 1</td>
<td>181.2</td>
<td>11.72</td>
<td>+41.9</td>
<td>2.13</td>
</tr>
<tr>
<td>CS-PEG-LAC/DNA</td>
<td>25</td>
<td>10: 1</td>
<td>276.83</td>
<td>9.28</td>
<td>+39.23</td>
<td>1.78</td>
</tr>
</tbody>
</table>

3.3 Proliferation of pig lymphoblasts in vitro

Firstly, we got TPIL-15 protein by transfection of HEK293 cell with the packed recombinant TPIL-15 plasmids in vitro, the transfection and gene expression efficiency were confirmed by reverse transcription PCR (RT-PCR). Bioactivity of TPIL-15 protein was detected by CCK-8. After using different nanoparticles to transfect HEK293 cells for 12h, 24h, 48h and 72h, supernatants of cell culture were used to stimulate the proliferation of pig lymphoblasts, and the results showed that the proliferation of pig lymphoblasts were increased by the CS derivatives (CS-PEG–PEI and CS-PEG-LAC) nanoparticles compared with control groups (Figure1), indicating that the molecular package with chitosan derivatives could promote the expression of TPIL-15 protein (P <0.05).
3.4 Immune responses of the mice in vivo

3.4.1 Immune cells in the peripheral blood of mice

During 35 days postinoculation, the growth rate of the experimental mice was significantly higher than control group (P<0.05), but after 21 days, the growth rates gradually slowed. There are no significant differences among CS, CS-PEG–PEI and CS-PEG-LAC treated groups (P>0.05) (Figure 2. a). As shown in Figure 2 b, c leukocytes and erythrocytes significantly increased in the experimental mice in comparison with the control group (P <0.05).
3.4.2 CD4+ and CD8+ T cells

20,000 leukocytes were randomly selected to count CD4+ and CD8+ T lymphocyte by FCM. The percentage and absolute value of CD4+ and CD8+ T lymphocyte were both significantly increased in the peripheral blood of the treated mice in comparison with those of the control mice from 21 to 35 days (P < 0.05); CD4+/CD8+ ratio remained relatively stable (approximate 2-2.5 folds) (P > 0.05) during the observation period (Figure 3. a, b).

3.4.3 Antibody and immunoglobulins

Figure 2.4.3 showed that the specific FMD antibody, IgG, IgG1 and IgG2a levels in the sera of the treated mice significantly increased than the control group (P < 0.05). The ratio of IgG1/IgG2a maintained at about 1.4. This shows that the mice were more likely to induce humoral immune response. CS, CS-PEG-PEI and CS-PEG-LAC package nanoparticle did not elicit significant differences among the treated groups (P > 0.05) (Figure 4. a-d).
3.4.4 Toll like receptors genes expression

Standard curve correlation co-efficient of TLRs primers testing were above 0.99, PCR amplification efficiency was 95%-105%. The results show that there were effective linear relationships PCR between the samples. TLRs were increased significantly during 0-35 days as showed in Figure 5 (P<0.05). TLR4 and TLR9 mRNA increased significantly more than other TLRs from 0-21 days. But TLR6 shows more cascade effect which elevated six times than the level before inoculation, and CS-PEG-PEI induced significantly higher responses than other materials (Figure 5. a-d).
3.4.5. Interleukins and TGF-β gene expression

Standard curve correlation co-efficient of interleukins and TGF-β primers testing were above 0.99, PCR amplification efficiency were 95%-105%. Transcription levels of IL-2, IL-4, IL-6 and IL-23 were significantly increased in the treated mice compared to those of the control mice during 35 days period (P <0.05) (Figure 6. a-e).
4. Discussion

The cationic polymers as CS, modified-CS and PEI have been considered as excellent candidates for preparation of nanoparticles\(^{15,18}\). Nanoparticles with specific size and positive charge on surface are necessary for endocytosis. Researches showed that many of proteins, drugs and DNA complexes were sensitive to bonding with cationic biomaterials delivery system; therefore, multi-cationic PEI was introduced onto CS to enhance the positive charge of the nanoparticles\(^{19,20}\). In our work, the average diameter of CS-PEG-PEI was smaller than packaged with CS which probably due to the increase of DNA binding/condensation capability. The zeta potential of CS-PEG-PEI was in the positive range and was higher than that of CS due to the introduction of polymeric PEI. As expected, with the increase of the mass ratio, the average diameter became smaller and smaller, and the zeta potential turned to be more positive, which suggest its better effectiveness for transfection and delivery of plasmids into cells.

In vivo experiment, CS-PEG-PEI/CS-PEG-LAC groups compared with CS/VR1020 group showed higher transfection efficency. Moreover, CS-PEG-PEI/CS-PEG-LAC nanoparticles were far less cytotoxic than PEI(data in our lab). There were also no detectable side effects in the weight gain, peripheral blood and the injection site of immunized mice.

It is recognized that IL-15/IL-15R signaling could modulate multiple functions of different immunocompetent cells, including T and B lymphocytes, NK cells, neutrophils and monocytes/macrophages of animals. IL-15/IL-15R is necessary for T lymphocyte development and homeostasis, and memory CD8\(^+\) T-cell and NK cell
development, maintenance, expansion and activities\textsuperscript{2,3,5,8}. Therefore, it was consistent with our findings that CD\textsuperscript{4}/CD\textsuperscript{8} T cell both increased in the peripheral blood of mice treated with packed VTPIL-15 nanoparticles.

Similarly, IL-15/IL-15R signaling affects B lymphocytes by activation of Syk kinase and PLC\textsubscript{γ}, protection from apoptosis, induction of proliferation and differentiation, and Ig production\textsuperscript{6,6,13}. Hence the results were found that the specific antibody to FMD vaccine and the IgG significantly increased in the sera of mice injected with IL-15 nanoparticles.

IL-15/IL-15R signaling can also remarkably elicit the activation of Jak2, Syk, p38, ERK1/2, NF-kB and down regulation of Bax, increase Mcl-1 protection from apoptosis, promote phagocytosis of mast cells, neutrophils and macrophages, which in turn enhance the phagocytosis for pathogen clearance and production of MCP-1, superoxide and interleukins\textsuperscript{6,6,14}. These changes could further increase expression of CD86, CD40 and MHC II in the immune cells that would amplify the relevant cellular and humoral immune responses\textsuperscript{6,9,12}. Besides, IL-15 can exert stimulatory effects on mast cells and dendritic cells by activation of STAT3, Jak2/STAT5,Tyk2/STAT6 and Syk, induce higher expression of Bcl-xL and c-Myc that lead to inhibition of apoptosis and support their proliferation and secretion of cytokines\textsuperscript{10,11,17}.

Based on our results, it is clear that the expressions of TRL1, TLR4, TLR6 and TLR9 genes were significantly increased by injection of recombinant IL-15 gene nanoparticles, which is closely associated with marked activation of non-specific immune cells, including monocytes /macrophages. TLR9 can specifically identify CpG-DNA sequence from bacteria and viruses. In additions, TLR9 can promote the ability of APC antigen-presenting to induce a variety of pro-inflammatory cytokines to speed up the defense reaction against infection of pathogens.

Interestingly, our results firstly reported that the expression of TGF-\textbeta and IL-23 genes were enhanced from 7 to 35 days after TPIL-15 nanoparticles injection; These prove that the entrapment of TPIL-15gene with chitosan and its derivatives is effective for gene delivery and transfection to elevate the innate and adaptive immunity of animals against pathogen. IL-23 is one of the critical cytokines for proliferation and development of immune memory cells (Bu/Tm)\textsuperscript{21}, it has a positive correlation with protective immunity. Therefore, TPIL-15 would not only potentiate immune responses of inoculated animals, but also facilitate the development of stronger immune memory capacity to prolong putative immunoprotection of vaccinated animals against reinfection.

In a word, TPIL-15 genes enwrapped with CS-PEG-PEI/CS-PEG-LAC is a promising and effective novel immunoadjuvant to improve the comprehensive immunity of animals to vaccine, which is worth of further exploration for preparation of safe and practicable molecular adjuvant.

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