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Construction and direct immunization of eukaryotic expression plasmid encoding human soluble B lymphocyte stimulator

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1. ABSTRACT

B lymphocyte stimulator of the tumor-necrosis-factor family (BLyS) enhances B cell survival, a function indispensable to B cell maturation. This factor plays a crucial role in enhancing immune responses. Here, to study the primary immune effect of BLyS gene in animals, we constructed recombinant eukaryotic expression plasmid of the human soluble BLyS by using expression vector pcDNA3.1 (-). The recombinant plasmid pcDNA3.1*BLyS* was injected subcutaneously into BALb/C mice and this administration induced the sustained expression of human BLyS and specific IgG against the recombinant BLyS protein. This study shows that recombinant eukaryotic expression plasmid, pcDNA3.1*BLyS,* might be used in human gene therapy.

2. INTRODUCTION

B lymphocyte stimulator (BLyS) (1) is a fundamental survival factor for transitional and mature B cells. The BLyS signaling is critically involved in B cell proliferation, maturation, and increases in serum immunoglobulin levels. It is a type II transmembrane protein, which can be expressed as a surface-membrane-bound molecule or secreted from cells as a soluble ligand (2).

Former studies show that both the membrane and soluble forms of BLyS are biologically active in promoting proliferation of B cells stimulated together with anti-IgM *in vitro* (1, 3). Animals injected with soluble recombinant BLyS exhibit the disrupted splenic T and B cell zones with

increased B cell counts and immunoglobulin levels. BLyS over expression in mice results in severe autoimmune lupus-like characteristics with high levels of rheumatoid factors, proteinuria, anti-DNA autoantibodies and increased numbers of B and effector T cells (4-8). Recent studies on BLyS gene knockout further reveal that BLyS is required for normal B lymphocyte development (9, 10). Taken together, such results demonstrate that BLyS plays a critical role in regulating B cell immune responses (11).

The identification of soluble BLyS may have therapeutic applications in B cell-related disease states such as single IgG and IgM deficiencies, common variable immunodeficiency (CVID) and other panhypogammaglobulinemias. In such conditions, abnormal B cell function leads to global or specific hypoimmunoglobulinemia and/or decreased secretory immunoglobulin levels. In many of these disease states, the treatment of choice is parenteral administration of immunoglobulin. However, B cell expansion and differentiation by means of BLyS treatment could provide a unique alternative to conventional therapy of parenteral immunoglobulin (12, 13).

In recent years cytokines have been implicated in the pathogenesis of tumoural, autoimmune and inflammatory diseases, and indicated as potential therapeutic targets. Blaese and his colleagues (14) transduced ADA gene mediated by RT-virus vectors into T lymphocytes of two children suffered from ADA-SCID, and observed rapid recovery of T cells counts and many cellular and humoral immune responses. Strom (15) transduced an oncoretroviral vector encoding WASP into primary human T cells derived from WAS patients and found that levels of WASP resulted in correction of the deficient proliferative response to T-cell receptor (TCR) stimulation characteristic of WAS and IL2 secretion after TCR stimulation was partially corrected. BLyS is a natural protein and can stimulate immunoglobulin secretion and decrease the body's susceptibility to virus infection. Since 1999, the BLyS protein has been the research target of scientists, but there was little report about the application of BLyS gene used for the therapy of CVID and other immunodeficiency disease. If administered BLyS gene can express normal BLyS protein and similarly promote the proliferation and differentiation of B cells, which will provide an economic, practical and long efficacy alternative to the conventional therapy of immunodeficiency disease.

Here, we constructed eukaryotic expression plasmid of BLyS, pcDNA3.1BLyS. By administering such plasmid into mice, the possibility of BLyS gene being developed into gene drug to substitute for BLyS protein in the therapy of hypoimmunoglobulinemia was investigated.

3. MATERIALS AND METHODS

3.1. Antibodies

Goat F (ab')$_2$ anti-human IgM was from Southern Biotechnology Associates Inc (Birminham, USA). For enzyme-linked immunosorbent assay (ELISA), the following materials were used: mouse IgG, IgM, goat anti-mouse HRP-IgG, goat anti-mouse HRP-IgM, (all from Sigma, St. Louis, MO, USA); goat anti-mouse IgG, goat anti-mouse IgM, mouse anti-goat HRP-IgG (Beijing Zhongshan Biotechnology, China); and goat anti-human BLyS from Santa Cruz biotechnology (Santa Cruz, CA, USA).

3.2. Plasmids construction

The full length cDNA encoding BLyS was inserted into the pBV220 constructing recombinant plasmid pBV*BLyS*. pBV*BLyS* was transformed into Escherichia *coli* DH5α, then expressed and purified. Next the obtained BLyS protein was confirmed by Western Blotting and N-terminal protein sequencing. Finally it was used for a series of functional identification in vitro and in vivo (16-17). Here, the purified recombinant BLyS protein was used for the next enzyme-linked immunosorbent assays.

For the construction of eukaryotic expression plasmids, the BLyS fragment was amplified by PCR from pBV*BLyS* and inserted into the eukaryotic vector pcDNA3.1 to form pcDNA3.1*BLyS* plasmid. The plasmids above were confirmed by standard techniques (18).

3.3. Enzyme-linked immunosorbent assay (ELISA)

Eighteen mice were randomized into 3 groups of 6 animals each. Mice were given pcDNA3.1, pcDNA3.1*BLyS*, or saline by subcutaneous injection on the rear at a dose of 100 μg each mouse with an interval of 10 days for once and three times in sum. On the day before every injection and two weeks after the last plasmid or saline injection, blood was collected from the tail vein and allowed to clot for 2 h at 4 °C, and then centrifuged at 12,000 rpm for 10 min at room temperature. Serum was separated, frozen at -20 °C, and later analysed for human BLyS, the type of anti-recombinant protein Ig, total serum IgG and IgM.

3.4. Statistical analysis

Statistical significance of differences was determined using the LSD post hoc test.

4. RESULTS

4.1. Production of the recombinant BLyS

It was proven that the pBV*BLyS* was highly expressed as inclusion body and the recombinant BLyS was expressed correctly by Western blotting (Fig. 1) and N-terminal protein sequencing. The purified BLyS was then used as coating material for the next ELISA.

4.2. Construction of eukaryotic expression plasmid of BLyS

The obtained *BLyS* fragments was inserted into pcDNA3.1 to form eukaryotic expression plasmid and verified by restriction analysis and sequencing.

4.3. Immunological effects of administered eukaryotic expression plasmid of BLyS on the expression of human BLyS, the type of Igs anti-recombinant protein and serum immnoglobulins in BALB/c mice

The competitive inhibition ELISA for the level of human BLyS in mice show that there was human BLyS

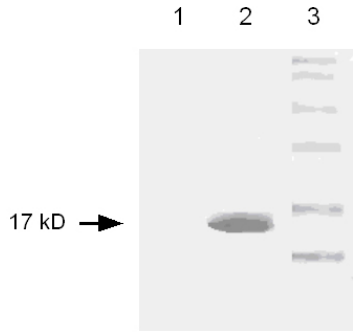

Figure 1. Western blotting analysis of recombinant BLyS. pBV220 (lane 1). pBV*BLyS* (lane 2). Low weight of protein marker (lane 3).

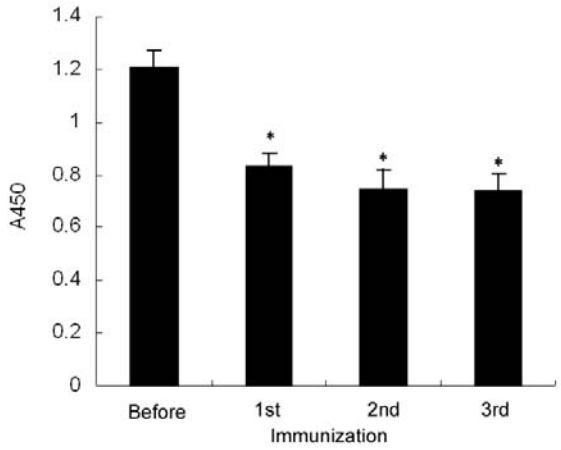

Figure 2. The expression of human BLyS after pcDNA3.1*BLyS* or controls administered into mice. The level of human BLyS was measured as absorbance (A450) values. *, Statistically significant differences ($p<0.05$) between the eukaryotic vector and controls.

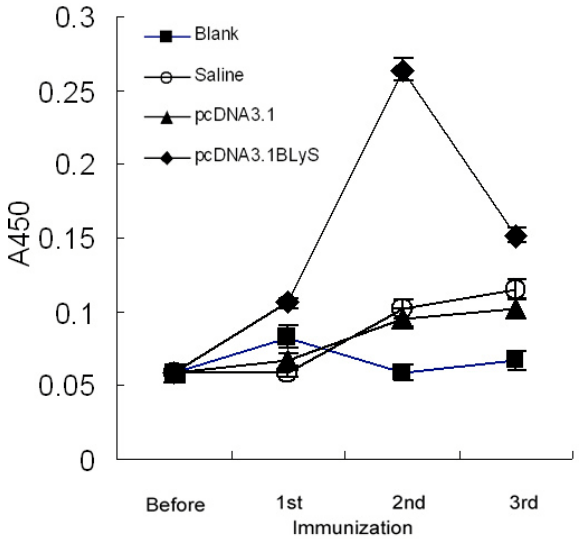

Figure 3. The level of IgG against the recombinant BLyS proteins. The level of IgG anti-BLyS was measured as absorbance (A450) values.

expressed after the first immunization, and the expression was durative even after the third immunization. Compared with the values of *A*450 before immunization, marked human BLyS expressed after three times of immunization (Fig.2). ELISA for the type of Igs against recombinant protein in mice indicated that IgG was the major type and there was no IgM being produced. Compared with the pcDNA3.1 and saline group, in the period of 3 times' immunization the *A*450 values of the anti-protein IgG in the eukaryotic expression vector group above ranked the highest after the second immunization and almost returned to basal level after the last immunization (Figure 3).

Compared with the pcDNA3.1 and saline group, the level of total serum IgG in the pcDNA3.1*BLyS* group displayed a little more higher after the second immunization and then decreased to the level as much as that of the saline group after the third immunization. However, statistical tests demonstrated that there was not much difference among the various groups (Figure 4). As for total serum IgM secretion, the mice of the pcDNA3.1*BLyS* group displayed no obvious increase whatever 2 or 3 times of immunization.

5. DISCUSSION

BLyS, a fundamental B cell survival factor, is being developed to increase the production of endogenous immunoglobulins in patients with hypogammaglobulinemia, such as CVID and IgA deficiency syndrome. So far, the main research target has been the wild type and there is only limited information about the derivatives of high activity but little side-effect of BLyS (17), saying nothing of BLyS gene being used for gene therapy in the treatment of CVID.

In recent years, a lot of related researches have been made about gene transfer. Demers (19) intravenously injected an E1 region-deleted adenovirus vector encoding human IFN-alpha2b gene driven by the cytomegalovirus immediate early promoter (rAd-IFN) into rats and rabbits to assess the serum concentration-time profiles of expressed IFN protein. Intravenous administration of rAd-IFN, normalized for body weight, resulted in dose-dependent serum IFN concentrations that persisted 8-40 days with similar concentration-time profiles in the animal models above. Similar serum IFN concentration-time profiles were obtained from two chimpanzees (approximately 70 kg) dosed with rAd-IFN by intravenous administration normalized to body weight. Later, the role of the immune response in limiting the persistence of transgene expression was highlighted by the persistence of serum IFN concentrations for over 200 days in beige/SCID immunodeficient mice. Ju (20) constructed eukaryotic expression plasmid of HSP65 gene from the H37Rv strain of Mycobacterium tuberculosis using pcDNA3.1(-). The recombinant plasmid pcHSP65 was transfected into HeLa cells by using the liposome transfection method and also injected into BALB/C mice to accomplish DNA immunization. Western blot varified the presence of a 65 kDa band of the expressed HSP65 protein and specific IgG

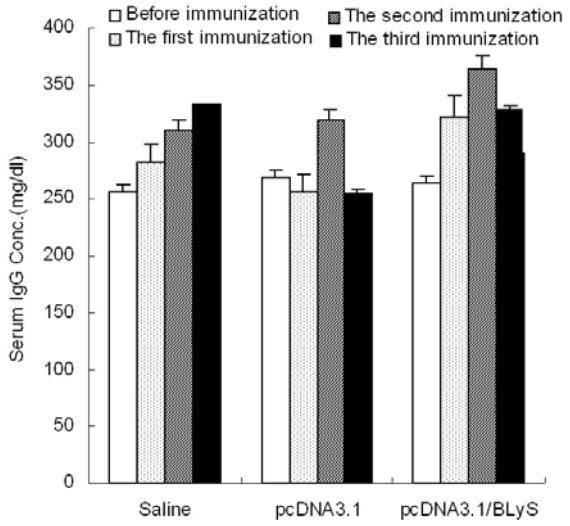

Figure 4. Effects of pcDNA3.1*BLyS* or controls administrations on the total serum IgG.

for the HSP65 protein could be identified in immunized mice. In this research, through competitive inhibition ELISA and indirect ELISA, repectively, we observed the lasting expression of human BLyS and determined specific IgG against the recombinant BLyS protein after the gene encoding BLyS was injected directly to BALb/C mice, which suggest that the recombinant plasmid pcDNA3.1/*BLyS* was successfully constructed and administered.

No obvious increases in total serum IgG and IgM secrection was observed, we presume when there is no outer stimulation, the steady and sustained expression of human BLyS after gene transfer might retain a stable environment for immune response *in vivo*, and only when stimulation comes could the expressed BLyS perform its normal biological function. Of course further work should be completed to further confirm these results.

6. ACKNOWLEDGEMENTS

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Abbreviations: BLyS, B lymphocyte stimulator; CVID, common variable immunodeficiency; ELISA, enzyme-linked immunosorbent assay; TNF, tumor necrosis factor

Key Words: Immune response, Blood cell, B Cell Proliferation. Immune deficiency, BLyS, Cytokine, TNF, ELISA, Immunoglobulin Secretion, Eukarotic Expression

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