Lentivirus-mediated soluble tumor necrosis factor receptor 1 expression in mouse bone marrow-derived immature dendritic cells**★

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Abstract

BACKGROUND: Tumor necrosis factor-α (TNF-α) is one of important cytokines to promote the maturation of dendritic cells. Blockage of TNF-α action by binding with soluble tumor necrosis factor receptor 1 (sTNFR1) may arrest dendritic cells in an immature state and induce stable, long-term tolerance.

OBJECTIVE: To construct the lentiviral vectors carrying sTNFR1 gene and investigate sTNFR1 expression in immature dendritic cells.

METHODS: Total RNA of human peripheral blood mononuclear cells was taken as a template. The sTNFR1 gene fragment was amplified by RT-PCR, subcloned to the lentiviral vectors pXZ208, and ligated to the enhanced green fluorescent protein (eGFP) reporter gene to establish lentiviral vector, called pXZ9-sTNFR1. DNA sequencing was performed for lentiviral vector identification. Lentivirus was prepared by transfection of 293 FT cells with pXZ9-sTNFR1. Viral titer was determined by eGFP expression. C57BL/6 mouse bone marrow-derived dendritic cells were *in vitro* cultured with low-dose granulocyte-macrophage colony stimulating factors and interleukin 4. On day 5 of culture, immature dendritic cells were transfected with pXZ9-sTNFR1 recombinant lentiviral supernatant. sTNFR1 transcription was detected by RT-PCR, sTNFR1 protein expression by Western blot analysis. Following sTNFR1 gene modification and lipopolysaccharide stimulation, the phenotype characteristics of dendritic cells were observed.

RESULTS AND CONCLUSION: Recombinant plasmid pXZ9-sTNFR1 was successfully constructed. Twenty-four hours after 293 FT cell transfection, eGFP expression was observed and viral titer was over 10^6 U/L. RT-PCR demonstrated that pXZ9-sTNFR1-transfected immature dendritic cells showed sTNFR1 positive expression. Western blot analysis revealed that sTNFR1 protein appeared in the immature dendritic cells and supernatant following 293 FT cell transfection. On day 5 of culture, dendritic cells expressed low level of class Ⅱ major histocompatibility complex (MHC Ⅱ), as well as CD40, CD86, CD80, molecules. However, following lipopolysaccharide stimulation, dendritic cells expressed high level of MHC II, as well as CD40, CD80, and CD86, molecules, exhibiting the phenotype characteristics of mature dendritic cells. But after sTNFR modification, the expression level of MHC II, as well as CD40, CD80, and CD86, molecules was not altered obviously. Lentiviral vectors carrying sTNFR1 gene and eGFP reporter gene were successfully constructed, and recombinant lentiviral plasmids with high titer were acquired. Following high efficacy of lentiviral gene transfection, immature dendritic cells stably express sTNFR1 mRNA and protein, which prevents immature dendritic cells from activation by exogenous lipopolysaccharide and maintains the immature state.

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INTRODUCTION

With the research progress in immunobiology, dendritic cells have been shown to play an important role in inducing and maintaining immunological tolerance. Dendritic cells are presently known the most efficient antigen presenting cells and are a kind of heterogeneic cell population with different subsets and different functions. Dendritic cells can cause the activation, differentiation, or tolerance of initial T cells. These different roles correlate with the maturating statuses of dendritic cells[1-3]. If the interaction of initial T cells and specific antigen peptide is accomplished by immature dendritic cells, then T cell inexcitability will take place, but under the condition of inflammatory cytokines, the inexcitability will turn into immunogenicity over the maturation of dendritic cells. Therefore, a key to induce immunological tolerance is to block the maturation of dendritic cells or to maintain the immature state[4-6].

The regulation of dendritic cell maturation involves tumor necrosis factor receptor or related signal transduction pathways. Tumor necrosis factor α (TNF-α) has been shown to exert a critical role in the maturation of dendritic cells sourced from bone marrow precursor cells and mononuclear precursor cells[7-8]. TNF-α biological function is accomplished by binding with cell surface TNF-α receptor. TNF-α receptors can be categorized into and membrane-bound and soluble TNF-α receptor (mTNFR, sTNFR, respectively). Under the activation of TNF-α converting enzyme, mTNFR extracellular region liberates from cell membrane, dissociates in the blood, and forms sTNFR compound. But sTNFR can not mediate TNF-α signal transduction[9-10], and thereby indirectly inhibit the physiological role of TNF-α. Therefore, a promising method to induce long-lasting immunological tolerance is to utilize TNF-α to resist sTNFR1 gene-modified dendritic cells and maintain their immature status[11-13].

The present study constructed recombinant lentiviral vectors carrying human sTNFR1 gene and used them to transfect mouse bone marrow-derived immature dendritic cells, so as to detect the phenotype and maturity status of immature dendritic cells.

MATERIALS AND METHODS

Design

An observational experiment based on gene level.

Time and setting

This study was performed at the Department of Hematology, Affiliated Hospital of Xuzhou Medical College from December 2007 to June 2009.

Materials

Four C57BL/6 mice of either gender, aged 6–8 weeks old, weighing (20±2) g, were purchased from Laboratory Animal Center, Xuzhou Medical College (Certification No. SCXK (su) 2003-0003). All experimental protocol was in accordance with *Guidance Suggestions for the Care and Use of Laboratory Animals*[11]. Human peripheral blood specimen was from a healthy volunteer blood donor, and written informed consent was obtained from the blood donor.

Methods

Primer design and synthesis

Referring to human sTNFR1 gene sequence from Genbank, primer was designed through the use of Primer Premier 5.0 software. According to enzyme digestion sites of lentiviral vector expressing pXZ208 and enzyme digestion site analysis atlas of human sTNFR1 gene sequence, *Bam*H Ⅰ and *Xho* Ⅰ enzyme digestion sites were inserted, and termination codon sequence was introduced. Upstream primer: 5'-TAG GAT CCT CTG GCA TGG GC-3'; Downstream primer: 5'-CCT CGA GCT ATG TGG TGC CTG AGT-3'

Cloning target gene from human peripheral blood mononuclear cells

sTNFR1 target gene was obtained by polymerase chain reaction (PCR), in which cDNA template was prepared by reverse transcription of human peripheral blood mononuclear cell total RNA (Trizol one-step protocol). PCR products were analyzed by agarose gel electrophoresis, retrieved, and purified using DNA extraction kit.

Reconstruction and identification of lentiviral vectors

sTNFR1 target gene was ligated to T vector pCR2.1, named pCR2.1-sTNFR1, and was identified by enzyme digestion. pCR2.1-sTNFR1 plasmid was digested using *Bam*HI and *Xho*I. sTNFR1 target gene fragment was retrieved, and ligated to linear vector pXZ208[12], forming pXZ208-sTNFR1. The purified IRES-eGFP target fragments were ligated to linear vector pXZ208-sTNFR1. The products were identified through a combination of *Eco*RI plus *Bgl*Ⅱ and *Bam*HⅠ plus *Xho*Ⅰ. The correctly ligated recombinant plasmid was named pXZ208-sTNFR1-IRES-eGFP (pXZ9-sTNFR1 for short).

Recombinant lentivirus package and titer detection

Through the use of liposome lipofectamine™ reagent, plasmids pXZ9-sTNFR1, pXZ9, △NRF, as well as envelope protein plasmid VSVG[13] were co-transfected into 293 FT cells in the exponential phase of growth. At 24 and 48 hours after transfection, enhanced green fluorescence protein (eGFP) expression was observed under the fluorescence microscope. At 48, 72, and 96 hours after transfection, virus-containing supernatant was collected, condensed, filtered, and preserved at −80℃ for future use. Following limiting dilution, the resulting products were observed under fluorescence microscope and viral titer was detected by flow cytometry.

Culture and identification of mouse bone marrow-derived immature dendritic cells

C57BL/6 mouse bone marrow cell suspension was routinely collected[14] and aliquoted into 6-well plate, approximately 1×10^6 cells per well. These cells were cultured with RPMI-1640 culture medium containing 20 μg/L granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 4 at 37 ℃ in 5% CO_2 environment. Cell growth was daily observed under the inverted phase contrast microscope. After 48 hours, culture medium and suspending cells were discarded, with exception of adherent cells. After fresh complete medium and the same concentration of cell factors were added, cells were cultured till day 5. Then lipopolysaccharide was, or not, used, cells were cultured till day 7, and suspending cells were collected. Following addition of CD16/CD32 antibodies, the collected cells were incubated for 30 minutes at 4℃ to block Fc receptor. Anti-mouse FITC-CD40, CD80, PE-CD86, CD11c and FITC-I-A/I-E monoclonal antibody were respectively added for 30-minute incubation. At the same time, homeotype controls were set. After incubation, there were two PBS washes. Cells were resuspended with 300 μL PBS for flow cytometry and Cell Quest Pro analysis.

Lentivirus infecting target cells and sTNFR1 detection

Immature dendritic cells cultured for 5 days were collected and placed in fresh culture medium containing GM-CSF and interleukin 4. Then 5 mL pXZ9-sTNFR1 and pXZ9 virus (control) were added. At the same time, Polybrene was added to make its final concentration 8 mg/L to promote viral adsorption. Normal bone marrow dendritic cells not-infected by virus were used as controls. After 96 hours of viral infection, cells were collected to observe eGFP expression under fluorescence microscope, and viral infection efficiency was detected by flow cytometry. The resulting cells were named sTNFR1-dendritic cells, pXZ9-dendritic cells, and day-5-dendritic cells, respectively. Dendritic cell surface marker in each group was analyzed by flow cytometry. sTNFR1 gene transcription in targeted cells was detected by RT-PCR. sTNFR1 protein level in target cells and culture supernatant were detected by Western bloat analysis.

Analysis of dendritic cell phenotype following lipopolysaccharide stimulation and sTNFR1 gene transfection

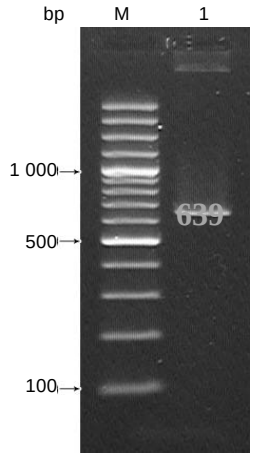
On day 5 of culture, sTNFR1-dendritic cells, pXZ9-dendritic cells, and day-5-dendritic cells were harvested and stimulated with 10 g/L exogenous lipopolysaccharide for 20 hours. Then, these cells were labeled with anti-mouse FITC-CD40, CD80, PE-CD86, CD11c and FITC- I-A/I-E monoclonal antibody, and cell phenotype was analyzed by flow cytometry.

RESULTS

PCR amplification of sTNFR1cDNA

cDNA obtained through human peripheral blood mononuclear

cell mRNA was taken as a template for PCR in which, *Bam*HI/*Xho*I enzyme digestion sites were introduced. An approximately size of 639 bp single, clear PCR band was obtained (Figure 1).

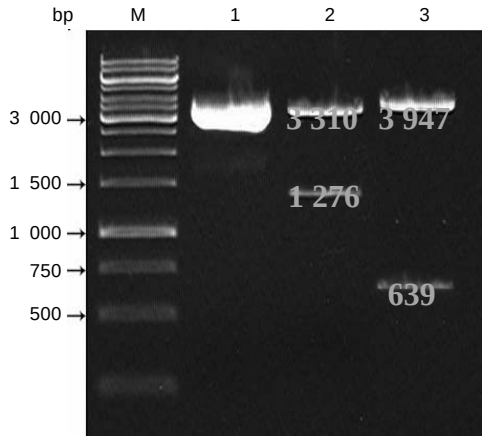


M: 100 bp DNA marker; Lane 1: sTNFR1 PCR

Figure 1 PCR amplification product of sTNFR1 cDNA

Identification of recombinant vectors

sTNFR1 target fragment was ligated to clone vector pCR2.1. After *Bgl*Ⅱ and *Bam*HⅠ/*Xho*Ⅰ digestion, fragments (1 276 bp + 3 310 bp and 639 bp + 3 947 bp) were obtained (Figure 2).



Lane M: 1Kb DNA marker; Lane 1: undigested plasmid; Lane 2: *Bgl*Ⅱdigested; Lane 3: *Bam*H/*Xho*Ⅰ digested

Figure 2 Electrophoretogram of plasmid sTNFR1-pCR2.1

Sequencing results confirmed that gene clone sequence in sTNFR1 region was correct. pXZ208-sTNFR1 expression vector was digested with *Eco*RⅠ+*Bgl*Ⅱ, or *Bam*HⅠ+*Xho*Ⅰ, and 1 160, 8 010 bp two fragments, as well as 639 bp, 8 531 bp two fragments were obtained, respectively. pXZ9-sTNFR1 expression plasmid was digested with *Eco*RⅠ or *Bam*HⅠ/*Xho*Ⅰ, and 8 872 bp+1 704 bp two fragments, as well as 9 937 bp+639 bp two fragments, were obtained, which were in accordance as expected(Figure 3).

Lentivirus package and viral titre determination

Through the use of liposome transfection, 293 FT cells were co-transfected using pXZ9-sTNFR1, pXZ9, △NR, and VSVG, 24 hours later, eGFP expression could be observed, and 50%–60% of cells were eGFP positive; 48 hours later, eGFP-positive cells were obviously increased, and eGFP positive rate was nearly 100% (Figure 4). eGFP expression was analyzed by flow cytometry. Limiting dilution assay results showed that viral titre was over 1×10^6 U/mL.

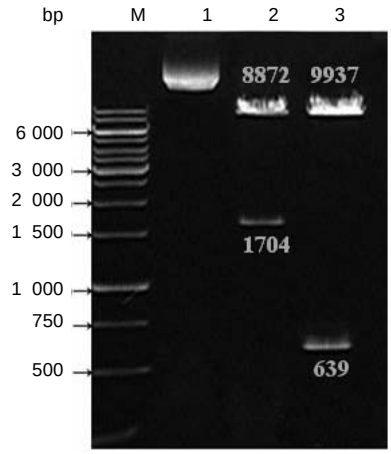
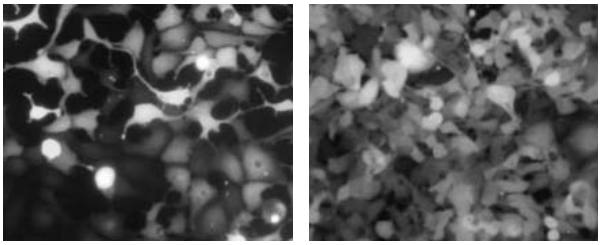


Figure 3 Electrophoretogram of enzyme digestion identification of constructed plasmid pXZ 9-sTNFR1



a: 24 hours post-transfection b: 48 hours post-transfection

Figure 4 Enhanced green fluorescence protein expression in 293 FT cells following viral transfection (× 200)

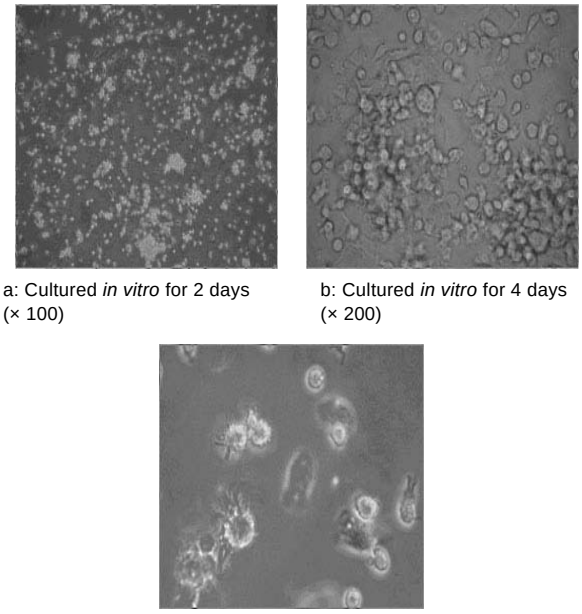
Amplification culture and identification of immature dendritic cells

After 24-hour culture of mouse bone marrow mononuclear cells, a large number of cells began to adhere to the wall (Figure 5a). At approximately 4 days, semi-adherent cell colonies appeared on adherent cell surface and gradually became more and enlarged over time (Figure 5b). Flow cytometry results showed that day-5-dendritic cells expressed low level of CD40, CD86, CD80, and major histocompatibility complex-Ⅱ (MHC Ⅱ) molecules and at 6–8 days, some cells presented with different lengths of bur-shaped processes, and single suspending typical dendritic cells could be observed (Figure 5c); day-8-dendritic cells expressed high level of CD40, CD86, CD80, and MHC-Ⅱ molecules.

sTNFR1 expression in dendritic cells following sTNFR1 gene transfection

pXZ9-sTNFR1 or pXZ9 was used to package recombinant lentivirus-transfected immature dendritic cells. Under fluorescence microscope, 60% of cells were observed with eGFP expression (Figure 6), as detected by flow cytometry

(Figure 7). Immature dendritic cells were transfected with sTNFR1 gene-carrying lentivirus, then RT-PCR yielded a 368 bp specific band, but no band was observed in the undigested cells (Figure 8). Western blot results showed that sTNFR1 protein existed in the supernatant of transfected immature dendritic cells, but not in the undigested cells (Figure 9).

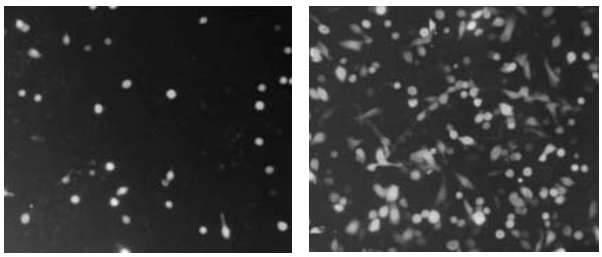


a: Cultured *in vitro* for 2 days (× 100)

b: Cultured *in vitro* for 4 days (× 200)

c: Cultured *in vitro* for 7 days (× 400)

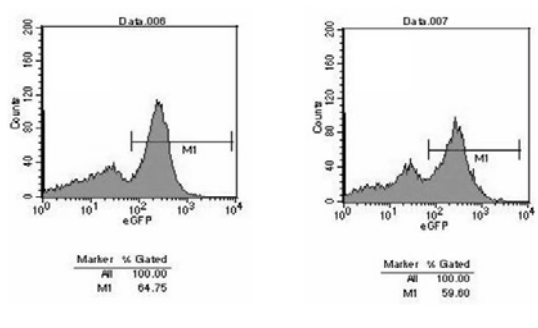
Figure 5 Morphological observation of dendritic cells at different culture stage



a: 24 hours post-transfection

b : 48 hours post-transfection

Figure 6 Enhanced green fluorescence protein expression in immature dendritic cells following viral transfection (×100)



A: sTNFR1-DC

B: pXZ9-DC

Figure 7 Detection of enhanced green fluorescence protein expression in immature dendritic cells 48 hours after transfection by flow cytometry

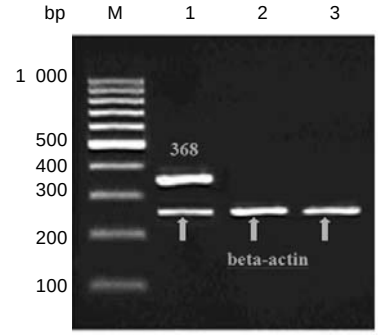
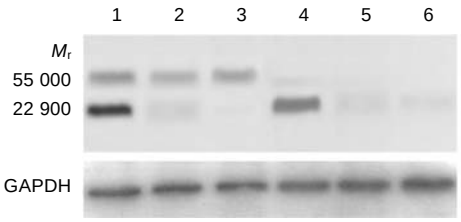


Figure 8 Detection of sTNFR1 gene expression in immature dendritic cells by RT-PCR



Lane 1: supernatant of sTNFR1-dendritic cell group; Lane 2: supernatant of pXZ9-DC group; Lane 3: supernatant of day-7-dendritic cell group; Lane 4: dendritic cells of sTNFR1-dendritic cell group; Lane 5: dendritic cells of pXZ9-dendritic cell group; Lane 6: dendritic cells of day-7-dendritic cell group

Figure 9 Detection of sTNFR1 protein expression in dendritic cells by Western blot analysis

Phenotype characteristics of dendritic cells following sTNFR1 gene transfection

sTNFR1 and pXZ9 gene modification produces no obvious influences on dendritic cell surface molecule expression. There was no obvious influence as compared with normal dendritic cells on day 5. But following lipopolysaccharide stimulation, day-7-dendritic cells or pXZ9-dendritic cells expressed high level of MHC Ⅱ molecules, as well as CD40, CD80, and CD86 molecules, exhibiting the phenotype characteristics of mature dendritic cells, but the expression level of MHC II molecules, as well as CD40, CD80, and CD86 molecules in the sTNFR1-dendritic cells was not obviously changed (Figure 10).

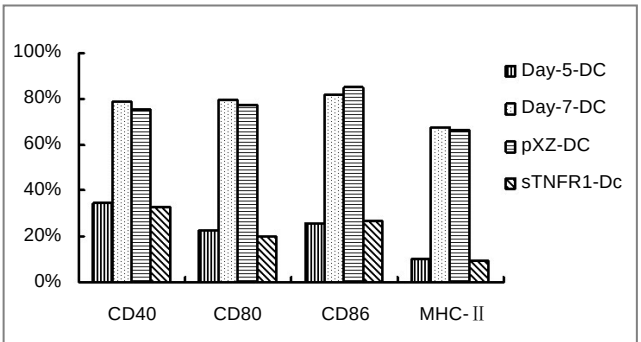


Figure 10 CD40, CD80, CD86, and major histocompatibility complex-Ⅱ (MHC-Ⅱ) expression in dendritic cells(DC) from different groups

DISCUSSION

Mature dendritic cells prone to immune activation, and immature dendritic cells prone to immune tolearance[15-17]. For mature dendritic cells, the ingested antigen binds to MHC Ⅱ molecules, and under the effects of synergic stimulation molecules, antigen peptide-MHC Ⅱ molecule complex binds to T cell surface TCR, and thereby to activate T cells. For immature dendritic cells, during the process of angtigen presentation, T cells can not be activated, and antigenic specific T cells inability is induced, due to lack of B7 molecules, thereby leading to antigen tolearance[18-19]. Great progress has been made to obtain tolerant dendritic cells. These methods include initial low-dose GM-CSF induction, classic GM-CSF plus interleukin 4 induction, and gene modification (transfecting transforming growth factor β, interleukin 10, sTNFR gene into mouse dendritic cells). These tolerant dendritic cells exhibit some advantages in inhibiting organ graft rejection and graft versus host disease[20-22]. Transformation of dendritic cells from immature precursor cells into mature cells is influenced by various factors, during which, changes in dendritic cell surface marker and function take place. Cytokine is an important factor that regulates the maturation of dendritic cells. An extremely small number of exogenous TNF-α added during *in vitro* culture can promote immature dendritic cells to develop into mature dendritic cells[7, 23-27]. Blocking TNF-α can inhibit the maturation and activation of dendritic cells. sTNFR gene, an antagonic molecule of TNF-α, can be used to modify tolerant dendritic cells and maintain their immature status, which is likely to be a more effective means to induce long-lasting immune tolerance[21]. At present, exogenous genes are transfected into dendritic cells through the use of viral vector systems, including adenoviral vector, adeno-associated viral vector, retroviral vector, and lentiviral vector. Our laboratory first successfully reconstruct a novel high titre inactivated lentiviral vector system in China, optimized internal promoters, and screened CMV promoter with highest expression efficiency[26]. The present study confirmed pCR2.1-sTNFR1 recombinant gene-engineered bacteria using enzyme digestion and nucleotide sequencing, compared sequencing factors with Genbank sequence, and obtained consistent results, without mutation. Then the pCR2.1-sTNFR1 was cloned into lentiviral transfer plasmid pXZ208, named pXZ9-sTNFR1. At 24 hours after transfecting 293FT cells, eGFP expression was observed under fluorescence microscope. Results demonstrated that viral titer exceeded 10^6 U/L, demonstrating that recombinant lentiviral expression vector carrying sTNFR1 gene was successfully reconstructed, and high viral titer was obtained. (60.37±5.48) % of immature dendritic cells could be transfected by viral supernatant, and sTNFR1 mRNA and protein expression was stable, providing experimental evidence for further studying the immune tolerance of sTNFR1 gene modified immature dendritic cells during transplantation. Some advancement has been presently made as to *in vitro* culture of dendritic cells. The amount of GM-CSF markedly correlates with maturation of dendritic cells cultured. Generally, high-dose GM-CSF primarily induce mature dendritic cells, while low-dose GM-CSF main induce immature dendritic cells[28-29]. 500–1 000 U/mL interleukin 4 can inhibit macrophage formation, and produce a synergistic effect with low-dose GM-CSF to enhance the amount and maturation degree of harvested cells[30]. The present study selected mouse bone marrow cells as the precursor cells of dendritic cells and used low-dose GM-CSF plus interleukin 4 to obtain higher purity of dendritic cells. This occurs possibly because GM-CSF produces proliferation-stimulating effects only on granulocytes, macrophages, and dendritic cells, while other cells, such as B cells and T cells, would gradually die during culture; granulocytes, the primarily contaminated cells, can not survive more than 10 days under the condition of 200 U/mL GM-CSF or lower concentration, and macrophages and other adherent cells would be separated in final cell harvesting. The present study also observed the development of dendritic cells. Results revealed that sTNFR1 gene could protect immature dendritic cells from activation by exogenous lipopolysaccharide, and compared with undigested immature dendritic cells, the phenotype of sTNFR1 gene-transfected immature cells did not change obviously. Exogenous lipopolysaccharide has been reported to promote dendritic cells to release TNF-α in the manner of autocrine and further promote the rapid differentiation and maturation of dendritic cells[31-32]. It is presumed that sTNFR1 gene modified immature dendritic cells maintain their immature status by local secretion of sTNFR1 to block the TNF-α autocrine by exogenous lipopolysaccharide-stimulated immature dendritic cells.

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慢病毒介导可溶性肿瘤坏死因子受体 1 在小鼠骨髓未成熟树突状细胞中的表达**★

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摘要

背景：肿瘤坏死因子 α 是介导树突状细胞成熟的重要细胞因子之一，可溶性肿瘤坏死因子受体1 与其结合可阻断肿瘤坏死因子α的作用，维持树突状细胞于不成熟状态，诱导免疫耐受。

目的：构建含有人 sTNFR1 的慢病毒表达载体，观察其在未成熟树突状细胞中的表达。

方法：以人外周血单个核细胞总 RNA 为模板，RT-PCR 扩增出 sTNFR1 基因片段，亚克隆至慢病毒转移质粒 pXZ208，通过 IRES 连接 eGFP 报告基因，建立双顺反子慢病毒转移质粒，命名为 pXZ9-sTNFR1，DNA 测序鉴定。采用脂质体转染 293 FT 细胞，根据报告基因 eGFP 测定病毒滴度。采用小剂量粒-巨噬细胞集落刺激因子+ 白细胞介素 4 体外培养扩增 C57BL/6 小鼠骨髓来源树突状细胞。培养第 5 天，以 pXZ9-sTNFR1 重组慢病毒上清感染未成熟树突状细胞，RT-PCR 检测感染后sTNFR1转录，Western blot 法检测 sTNFR1 蛋白表达，观察 sTNFR1 基因修饰及脂多糖刺激后树突状细胞的表型特征。

结果与结论：成功构建重组质粒 pXZ9-sTNFR1，转染 293 FT 细胞 24 h 后观察到 eGFP 表达，病毒滴度在 10^6 U/L 以上。RT-PCR 显示 pXZ9-sTNFR1 感染的未成熟树突状细胞 sTNFR1 呈阳性表达，Western blot 检测到 sTNFR1 蛋白存在于感染后未成熟树突状细胞和培养上清中。培养第 5 天的树突状细胞低表达 CD40、CD86、CD80 和 MHCⅡ类分子，脂多糖刺激后，高表达 MHCⅡ类分子和 CD40、CD80、CD86 分子，显示出成熟型树突状细胞表型特征，sTNFR 修饰的树突状细胞 MHCⅡ类分子和 CD40、CD80、CD86 分子表达水平无变化。

提示：①成功构建了负载 sTNFR1 基因片段及含 eGFP 报告基因的慢病毒载体，获得了高滴度的重组慢病毒颗粒。②经慢病毒高效转导的未成熟树突状细胞 sTNFR1 mRNA 及蛋白稳定地表达，可以保护未成熟树突状细胞不被外源性脂多糖刺激活化，维持树突状细胞于非成熟状态。

关键词：可溶性肿瘤坏死因子受体；基因修饰；慢病毒载体；未成熟树突状细胞；免疫耐受

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