PREPARATION AND IDENTIFICATION OF TNF-A ANTI-IDIOTYPIC ANTIBODIES WHICH CAN BE USED TO DETECT AND TREAT INFLAMMATORY-RELATED DISEASES

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ABSTRACT

Objective*: Tumor necrosis factor (TNF-α) is mainly produced by macrophages and monocytes, it is a potent pro-inflammatory and pathological cytokines in inflammatory diseases. TNF-α is closely related to a series of diseases (such as inflammatory bowel diseases), therefore anti-TNF-α or anti-TNF-α receptor (TNFR) therapy strategy has been established as an efficacious therapeutic strategy in these diseases.*

Methods*: In the current research, we used anti-idiotypic antibody strategy to prepare a TNFR antagonist. A series of biological technologies (such as Western-blot and indirect immunofluorescence assay) were used to screen and identify TNF-α anti-idiotypic antibody (4G-89).*

Results: we found that the anti-idiotypic antibody 4G-89 can specifically compete with TNF-α binding to TNFR. 4G-89 also can be used to detect the expression level of TNFR. In addition, we also found that the anti-idiotypic antibody has the potential to treat the inflammatory-related disease induced by TNF-α.

Conclusions*: The current study showed that anti-idiotypic antibody may be a potential strategy for the preparation of TNFR antagonists, which laid the foundation for development of TNFR antagonist in the future.*

Keywords: *NFα, Anti-idiotypic antibody, Antagonist, inflammatory-related diseases.*

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Introduction

Tumor Necrosis Factor α (TNF-α) is a type of cytokine with many biological effects[(1)]. After binding to specific receptors on the cell membrane, TNF-α exhibits its biological effects (such as cell differentiation, apoptosis and inducing inflammation). TNF-α displays its biological function by interacting with TNFR expressed on the cell surface. There are two types of TNFR: TNF-R1 (55 kD) and TNF-R2 (75 kD), both of which belong to type I membrane proteins, consisting of signal peptide, extracellular domain, transmembrane domain and intracellular domain[(2)].The homology of the intracellular domains of TNF-R1 (55 kD) and TNF-R2 is low. TNF-R1 contains the death domain (DD), while TNF-R2 does not contain DD, which implies that the two types of receptors activate different signaling pathways and mediate different biological functions .

TNF-α is mainly produced by activated monocytes and macrophages[(2)]. It can not only induce apoptosis of a variety of cancer cells, but is also related to the pathogenesis of inflammatory diseases and autoimmune diseases[(3-4)]. The imbalance of TNF-α signaling is the main cause of the various inflammations and autoimmune diseases, such as inflammatory bowel disease, rheumatoid arthritis

and psoriasis[5]. Therefore, blockage of TNFR can treat certain inflammation-related diseases. So far, five biological agents (drugs) have been approved for the treatment of TNF-α-related diseases. They are infliximab (Remicade), adalimumab (Humira), golimumab (Simponi), Nasicept (Enbrel) and Certuzumab pegylation (Cimzia)[6], they can neutralize or inhibit the TNF-α's activity. Another strategy to block TNFα/TNFR is to prepare TNFR antibodies. Although these therapies have been shown to be effective in treating inflammatory diseases, but many adverse reactions have been reported, such as psoriasis and malignant tumors[7].

In the current research, we prepared an anti-idiotypic antibodies against TNF-α (termed as 4G-89) by anti-idiotypic antibody strategy. After a series of identifications, we found that 4G-89 could inhibit the TNF-α/TNFR-mediated biological activity, indicating that anti-idiotypic antibody of TNF can be used as an antagonist of TNFR. The current study showed that anti-idiotypic antibody may be a potential strategy for the preparation of TNFR antagonists.

Materials and methods

Materials and reagents

All animal care and experimental procedures were approved by the first affiliated hospital of Nanjing Medical University. Mouse myeloma cell line (SP 2/0) was purchased from Shanghai Chinese Academy of Sciences Cell Bank. Female SPF Balb/c mice aged 8-10 weeks were obtained from Hua-Fukang company. TNF-α was purchased from Sigma Company. Hypoxanthine H, aminopterin A, and thymidine T (HAT) were purchased from Thermo fisher; Freund's complete adjuvant and Freund's incomplete adjuvant were purchased from Sigma; cell fusion agent polyethylene glycol was purchased From Sigma.

Cell culture

LO2 liver cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) in an incubator at 37°C and 5% CO2. The cells were subcultured with 0.25% trypsin.

Preparation of Anti-TNFα antibody

We first prepared rabbit anti-TNF-α polyclonal antibodies. TNF-α was emulsified with an equal volume of complete Freund's adjuvant, and the mixture was injected intradermally into 4 separate sites on the back of the neck of the rabbits. The booster immunizations were repeated every 2 weeks. After three immunizations, serum antibody titer was assessed by ELISA assays. 10 days after last booster immunization, the blood was collected from the heart, and the serum was separated and stored at -20°C for future use.

Preparation of TNF-α anti-idiotypic monoclonal antibody

Anti-TNF-α was used as an immune antigen to immunize Balb/c mice (multi-point subcutaneous injection immunization), the dose was 200 μg per mouse. booster immunization was performed at two-week intervals. When the serum titer reached 1×104. The last booster immunization was done. Three days after the booster immunization, mouse spleen cells were collected. spleen cells and SP 2/0 cells were fused with PEG4000 in a ratio of 10:1. ELISA was used to screen positive clones.

ELISA

The hybridoma cell culture supernatant was screened by the indirect ELISA. TNF-α (1ug/mL) was added into the 96-well microtiter plate (100μL per well) overnight at 4°C. After washing with PBS-Tween 20, 200μL blocking solution (3% BSA) was added into each well at 37°C for 2 h. The culture supernatant of the hybridoma cells were added overnight at 4°C. After washing for three times with PBS-Tween 20, 100 μL secondary antibodies were added to each well (diluted 1:4000) at 37°C for 2 h. After washing for three times, Color was developed by adding TMB.

Production of monoclonal antibodies in vivo

Firstly, BALB/c mice were sensitized by intraperitoneal injection of 0.5 mL liquid paraffin. After 7 days, the 5.0×106 hybridoma cells were injected into the abdominal cavity (0.5 mL per mouse). After 10-14 days, the mouse ascites was collected, and the antibody was purified by saturated ammonium sulfate salting. The purified antibodies were store at -20°C.

Indirect immunofluorescence

The LO2 cells were incubated with TNFα or 4G-89 for 30 min, after which, the cells were washed three times. After the cells were blocked with 3% BSA for 2 h, the secondary antibody was added and incubated for 1 h. After washing, the cell samples were observed by a confocal microscope (Olympus

FV3000).

Western-blot

The total cell protein was extracted with RIPA lysate, and the protein concentration was detected by the BCA method. The samples were then subjected to SDS-PAGE gel electrophoresis and transferred to PVDF membrane. After washing for three times with washing buffer (PBS, pH 7.4, 0.01 M, containing 0.05% Tween 20), the indicated primary antibody was added and incubated overnight at 4°C. After washing with PBST, the secondary antibody (diluted 1:5000) was added and incubated for 2 h. After washing for three times, blots were visualized with an enhanced chemiluminescence kit. Image J software was used to measure the gray value of the target band.

Competitive receptor binding analysis

The LO2 cells were incubated with TNFα and increased concentrations of 4G-89 (or control antibody) for the indicated time points. After washing, the cell samples were analyzed by Flow cytometry.

Flow cytometry analysis

1×106 LO2 cells were collected, the cells were then washed three times with 1 ml FACS buffer (1% BSA-PBS), and centrifuged at 5000 r/min for 5 min. The supernatant was then discarded. After the cells were fixed with 4% paraformaldehyde, the indicated primary antibody was added and incubated at 4°C for 0.5 h in the dark. After washing with 1 ml FACS buffer, the cell samples were detected by FACS Calibur Flow Cytometer.

Statistical analysis

The data are reported as the mean ± standard error (SE). The data were analyzed using a one-way analysis of variance (ANOVA). Statistical analysis was performed with SPSS software (version 20.0, SPSS Inc., Chicago, IL). A p-value of <0.05 was considered statistically significant.

Results

Screening and identification of TNF-α anti-idiotypic antibodies (4G-89)

In the current study, we first prepared polyclonal antibodies against TNF-α (anti-TNFα). Then we used anti-TNF-α as the antigen to immunize mice to prepare anti-idiotypic monoclonal antibodies. After a series of identifications, we identified a TNF-α anti-idiotypic antibody with antagonistic activity (named 4G-89, IgG2A). To further analyze 4G-89, the competitive ELISA experiments were performed, and results showed that 4G-89 could inhibit anti-TNF-α binding to TNF-α (Figure 1A). Similarly, further ELISA assays showed that TNF-α competed with 4G-89 to bind anti-TNF-α (Figure 1B).

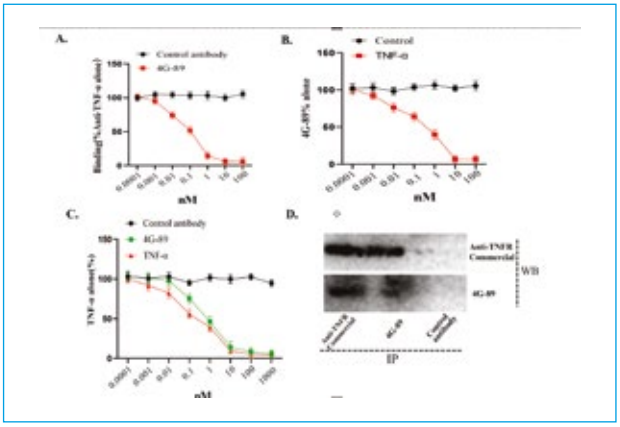

Fig. 1: Identification of anti-idiotypic antibody (4G-89). **A**. 4G-89 inhibited anti-TNF-α to bind to TNF-α in a dose-dependent manner. **B**. TNF-α competed with 4G-89 to bind anti-TNF-α. **C**. Competitive receptor binding analysis shows that 4G-89 can competitively bind to TNFR with TNF-α. **D**. IP-WB analyzed the interaction between 4G-89 and TNFR. E. Co-localization analysis of the interaction between 4G-89 and TNFR.

In summary, competitive ELISA assays showed that 4G-89 can mimic an epitope on TNF-α, suggesting that 4G-89 has the typical Ab2β characteristics. To further verify whether 4G-89 can bind to TNF-α receptors, competitive receptor binding analysis was used. As we can see in Figure 1C, 4G-89 could competitively bind TNFR with TNF-α in a dose-dependent manner.

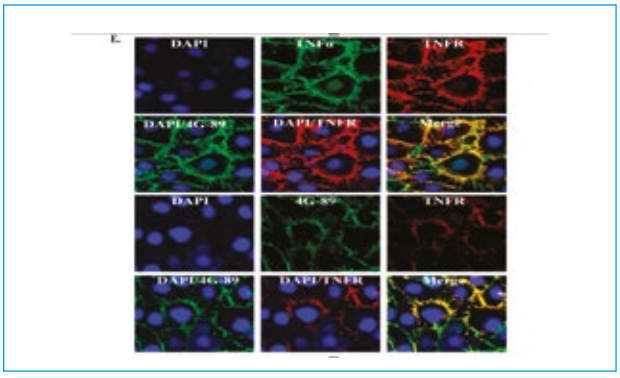

Fig. 1: **E**. The interaction between 4G-89 withTNFR by CLSM analysis.

Furthermore, co-immunoprecipitation and colocalization have also been done, and results further demonstrated that 4G-89 can specifically bind TNFR. Affinity analysis showed that the affinity of 4G-89 was 1.5×10-8mol/L for TNFR (Figure 1 D and E).

4G-89 can be used to detect TNFR expression

In addition, we initially determined whether 4G-89 could detect the expression of TNFR1 in hepatocytes models. For this, Flow cytometry and indirect immunofluorescence experiments were performed, and the results showed that 4G-89 could recognize TNFR (Figure 2A). These findings suggest that 4G-89 has potential application for evaluation of TNFR at the cell or tissue level (Figure 2B).

Figure 2 A. 4G-89 could recognize TNFR as determined by Flow cytometry. B. 4G-89 has potential application for evaluation of TNFR expression. Asterisk (*) represents a statistically significant (P<0.05). Data represent the mean ± SD of three biological replicates.

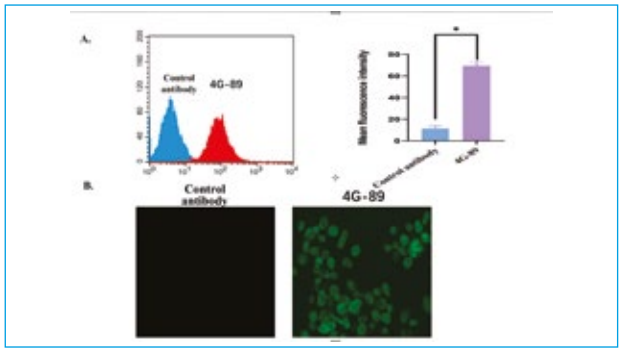

Fig. 2: **A**. 4G-89 could recognize TNFR as determined by Flow cytometry. B. 4G-89 has potential application for evaluation of TNFR expression. Asterisk (*) represents a statistically significant (P < 0.05). Data represent the mean ± SD of three biological replicates.

The effect of 4G-89 on TNF-α-mediated signaling transduction

We analyzed the effect of 4G-89 on TNFα-mediated signal transduction by flow cytometry. As shown in Figure 3, the results showed that 4G-89 inhibits the signaling pathways induced by TNFα in a time-dependent manner (Figure 3).

Figure 3. 4G-89 inhibits the signaling pathways induced by TNFα in a dose-dependent manner. The detailed steps of the experiment have been described in detail in Materials and Methods. Asterisk (*) represents a statistically significant (P<0.05). Data represent the mean ±SD.

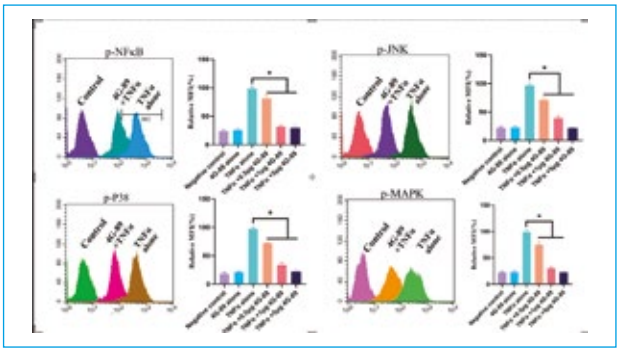

Fig. 3: 4G-89 inhibits the signaling pathways induced by TNFα in a dose-dependent manner. The detailed steps of the experiment have been described in detail in Materials and Methods. Asterisk (*) represents a statistically significant (P< 0.05). Data represent the mean ±SD.

Effects of 4G-89 on cell viability

Here, we further tested the potential biological activity of 4G-89. Liver cell damage models was established by GalN/TNF-α treatment according to previous methods. As expected, we can see that GalN/TNF-α significantly inhibited the survival of LO2 cells. However, when the cells were pretreated with 4G-89 (0.2, 0.5 and 1 $\mu g \cdot mL^{-1}$), the cell viability was increased compared with control group (Figure 4A).

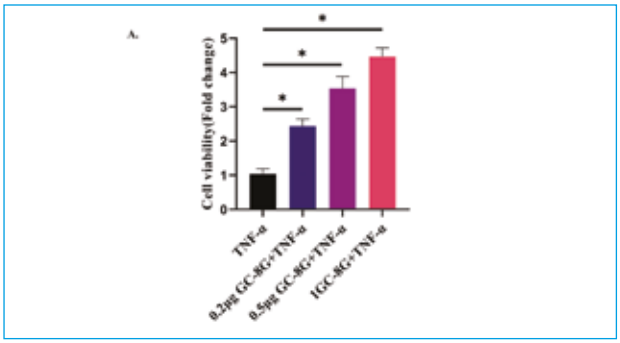

Fig. 4: **A**. 4G-89 increased the cell viability compared with control group.

In addition, GalN/TNF-α was used to induce liver damage models in vivo. As we can see that 4G-89 (but not control antibody) could significantly relieve the inflammation induced by GalN/TNF-α (Figure 4B). In addition, we also found that 4G-89 (but not control antibody) inhibited the expression of proinflammatory factor IL-8, MCP-1 (Figure 4C).

Effects of 4G-89 on GalN/TNF-α-induced apoptosis

Flow cytometry was done to analyze the effects 4G-89 on GalN/TNF-α-induced apoptosis.

As indicated in Figure 5A, GalN/TNF-α treatment increased the rate of cell apoptosis (P <0.05), when the cell was treated with 4G-89, the LO2 apoptotic rates was significantly decreased compared with the

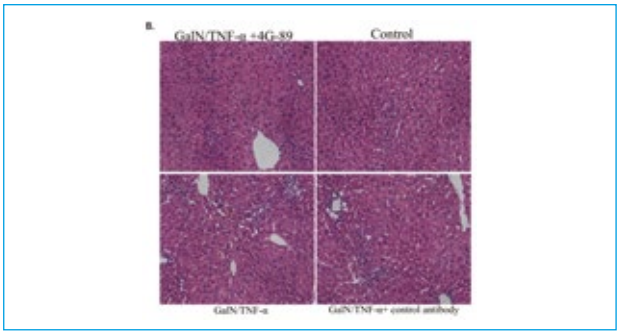

Fig. 4: **B**. 4G-89 (but not control antibody) could significantly relieve the inflammation damage caused by GaIN/ TNFα

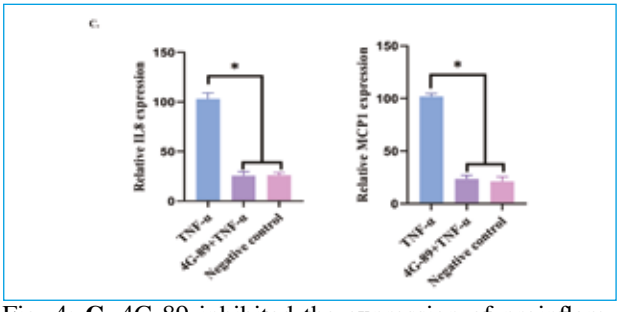

Fig. 4: **C**. 4G-89 inhibited the expression of proinflammatory factor IL-8, MCP-1. Data represent the mean ± SD of three biological replicates. Asterisk (*) represents a statistically significant (P <0.05).

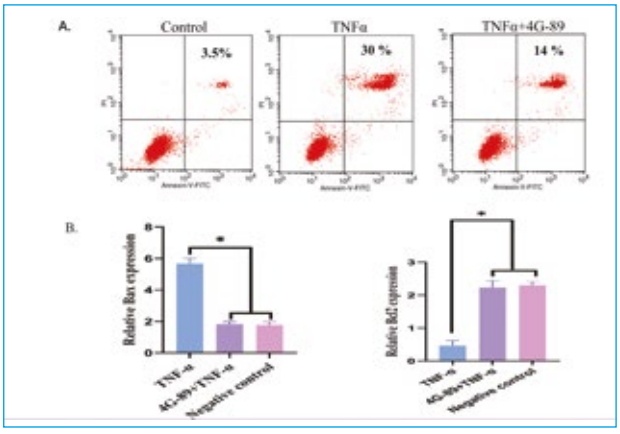

Fig. 5: **A**. 4G-89 decreased LO2 apoptosis induced by GalN/TNFα. **B**. 4G-89 could down-regulate bax expression, and up-regulate Bcl-2 expression. Asterisk (*) represents a statistically significant (P<0.05). Data represent the mean ±SD.

GalN/TNF-α alone treatment group. Taken together, these results suggested that 4G-89 protects cells from GalN/TNF-α-induced apoptosis. Because the Bcl-2 displays an anti-apoptotic effect and Bax promotes apoptosis, we investigated the effect of 4G-89 on the expression of Bax and Bcl-2 in the model of GalN/ TNF-α-mediated hepatocyte damage model. Compared with the normal hepatocytes, GalN/TNF-α-treated cells showed an obvious increase in Bax expression and a decrease in Bcl-2 expression, which was reversed by 4G-89 treatment (Figure 5B). These findings further indicated that 4G-89 has a protective effect against TNFα-induced apoptosis.

Conclusion

The immune system is a complex system which protects against invaders and maintains tissue homeostasis[9]. It is composed of immune tissue, immune cells and immune molecules. The body's immune function includes humoral immunity and cellular immunity. Antibodies are the most important component of humoral immunity. Due to the different genetic basis of antibody production, there are differences in the antigenicity of different antibody molecules. Serological methods can be used to determine and analyze the antigenicity of different antibody molecules and classify them. This is called the antibody serotype (immunoglobulin Serotype). Antibody serotypes can be divided into isotype, allotype and idiotype (Idiotype, Id). Anti-Id is divided into four categories: Ab2α, Ab2β, Ab2γ, and Ab2δ[10-12]. Among them, Ab2β can mimic the epitope of the original antigen, so it is called "internal image" of initial antigen. In the current research, we prepared an anti-idiotypic antibody to TNF-α (termed as 4G-89) through anti-idiotypic antibody strategy. After a series of identifications, we firstly demonstrated that 4G-89 could bind to TNFR, the results form competitive receptor binding analysis, co-immunoprecipitation and colocalization also demonstrated that 4G-89 could specifically bind TNFR (Figure 1). We further analyzed the potential biological activity of 4G-89, and found that it can be used to detect the TNFR expression (Figure 2). More importantly, 4G-89 can also inhibit the biological activity of TNF-α/TNFR. The results of flow cytometry showed that 4G-89 could inhibit TNFR-mediated signaling pathway. Taken together, the current research shows that 4G-89 was a new TNFR antagonist.

TNF-α not only can induce apoptosis of a variety of cancer cells, but also is related to the pathogenesis of inflammatory diseases and autoimmune diseases(10). Therefore, blocking TNF-α can treat certain inflammation-related

diseases. In the current study, hepatocytes damage models were established by GalN/TNF-α treatment. As expected, GalN/TNF-α significantly inhibited the survival of LO2 cells. However, when the cells were pretreated with 4G-89 (but not control antibody), the cell viability was increased compared with control group (Figure 4A), suggesting 4G-89 could inhibit TNFR's biological activity. Next, GalN/TNF-α was also used to establish liver damage model in vivo, after which, 4G-89 was used, and results showed that 4G-89 could alleviate the liver damage induced by GalN/TNF-α. We further study the effects of 4G-89 on expression of IL8 and MCP-1, and results showed that 4G-89 can inhibit TNF-α-induced inflammatory damage. In addition, Flow cytometry was done to analyze the effect of 4G-89 on GalN/TNF-α-induced apoptosis, and results showed that 4G-89 can alleviate the cell apoptosis induced by GalN/TNF-α treatment.

In the current study, we have prepared an anti-idiotypic antibody against TNF-α through a new strategy (anti-idiotypic antibody strategy). After a series of identifications, we found that 4G-89 can not only specifically bind to TNFR, but also inhibit the biological activity mediated by TNFα/TNFR. In summary, it is expected to be used in clinical applications in the future. 4G-89 may have excellent application potential. This work also shows that the anti-idiotypic antibody strategy may be a good strategy for preparing antagonists of TNFR.

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