De novo generation of specific human IgGs by in vitro immunization using autologous proteins containing immunogenic p-nitrophenylalanine

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ABSTRACT

In vitro immunization can be used to produce monoclonal antibodies (mAbs), but this technology is limited by poor reproducibility and the requirement of pre-immunized lymphocytes. To improve this approach, we recently developed a method for rapid generation of antigen-specific B cells. Here, we report the application of this systemic approach to the production of human IgGs against tumor necrosis factor (TNF). We expressed mutant proteins with site-specific incorporated p-nitrophenylalanine (pNO2Phe), which stimulated an in vitro immune response in human immune cells. After constructing an antigen-specific antibody library from in vitro immunized B cells identified by fluorescence-activated cell sorting, we demonstrated that many point mutation events of the variable region occurred in our step-by-step co-cultivation system for affinity maturation in vitro. To mimic the class switching, we panned for high-affinity antigen-binding fragments by the phage display method, assembled them and identified hTNF-neutralizing human IgGs. This approach may provide a general method for raising high-affinity monoclonal antibodies against self-proteins. Furthermore, it supports mechanistic understanding in breaking human self-tolerance with pNO2Phe.

Introduction

Monoclonal antibodies (mAbs) are a valuable class of biologics used as pharmaceuticals and diagnostics. Many technologies exist for the generation of mAbs, including transgenic mice with human antibody repertoires, phage display method or the immortalization of human B cells. These methods, however, have several drawbacks. Thus, the recovered antibodies might not display the precise specificities of antibodies in human immune system. The phage display system requires fixed libraries of high quality and enormous size, and unnatural VH and VL antibody pairings may result. The immortalization of human B cell is unsuitable for a comprehensive screening. Transgenic mice systems cannot precisely imitate a human immune response. In vitro immunization was used by different groups to produce human mAbs against several targets, including proteins, bacteria, and cells. In vitro immunization with antigens and cytokines triggers specific human naive B-cell response in short periods, but the approach has not been used widely, primarily because the procedures are complicated and can be repeated only in a few cases. Recently, we developed a method for the rapid generation of antigen-specific B cells to improve in vitro immunization and make protocols simpler. Our system has proved to be an effective method for the de novo acquisition of antigen-specific B cells of interest.

In addition to the model antigen, Keyhole limpet hemocyanin (KLH), we wondered whether this method can be used on autologous proteins for generation of more meaningful therapeutic mAbs. However, immunological tolerance of self-proteins is a big obstacle for in vitro immunization. In previous studies, the site-specific incorporation of the unnatural amino acid p-nitrophenylalanine (pNO2Phe) into the self-proteins was shown to induce long-lived auto-antibody responses to their respective endogenous counterparts in vivo. Furthermore, introduction of pNO2Phe into a protein sequence could create a strong CD4+ T cell epitope, without affecting the overall quaternary structure of the protein. In addition, pNO2Phe-containing self-antigens induced a protective cross-reactive immune response without the need for strong adjuvants and resulted in high titers for at least 4 months in mice. Thus, we decided to assess whether endogenous protein variants containing pNO2Phe are able to trigger a humoral immune response in vitro.

In this study, we aimed to establish a universal method that allowed de novo generation of specific human IgGs. Based on this method, we chose human tumor necrosis factor (hTNF) as the target protein and expressed variants with site-specific incorporation of pNO2Phe. Furthermore, we verified that these mutant proteins could trigger an in vitro immune response in human immune cells. After constructing an antigen-specific antibody library of small size from in vitro immunized B cells identified via fluorescence-activated cell sorting, we compared the diversity of the antibody variable region with germline genes. Many point mutation events of the variable region occurred in our step-by-step co-cultivation system for affinity maturation in vitro. In order...
to mimic the class switching, we panned for high-affinity antigen-binding fragments by the phage display method, assembled them and a hTNF-neutralizing antibody was identified.

**Results**

**Production of wild-type and mutant proteins**

We selected hTNF as the target protein for this study because its structure-activity relationship has been extensively studied. Based on the x-ray crystal structure of trimeric hTNF, a solvent-exposed residue (Tyr87 or Lys11) was selected for pNO2Phe incorporation. The mutant sites are indicated in the crystal structure (Figure 1a; Protein Databank ID code 1TNF). These two residues were selected because their mutations could eliminate cellular activity but not trimer formation. Wild-type and mutant hTNF proteins were expressed in E. coli cytoplasm as the soluble form. No full-length hTNF was observed when the mutant gene was expressed in the absence of pNO2Phe, indicating that pNO2Phe-specific tRNA/tRNA synthetase pair is orthogonal to other endogenous pairs (Figure 1b). Following chromatographic purification, each hTNF recombinant protein has an apparent molecular mass of 18 kDa, which produced a single band (purity: >90%) as determined by SDS-PAGE (Figure 1c). The mass spectrometry (MS)/MS analysis of tryptic fragment exactly matched the pattern for the incorporation of pNO2Phe at residue 87 and 11, respectively (Table 1, Fig S1).

**Activation of naïve CD4+ T cells primed by autologous antigens-loaded dendritic cells**

To determine the immunogenicity of different pNO2Phe-containing protein mutants, we tested the proliferative response of naïve CD4+ T cells primed by these variants-loaded dendritic cells (DCs). DCs pulsed with native hTNF could not increase the T-cell proliferation (Figure 2), suggesting that CD4+ T cells remain tolerant to endogenous proteins. In contrast, all hTNF variants-loaded DCs generated a large population of activated T cells, which increased with DCs/T cell ratio (Figure 2). Moreover, the site of pNO2Phe incorporation affected T-cell activation (Figure 2). Based on these results, pNO2Phe87hTNF with the highest immunogenicity was selected as a candidate for subsequent in vitro immunization studies.

**Follicular helper T cell differentiation of naïve CD4+ T cells**

As we have previously elicited in vitro follicular helper T (Tfh) cell differentiation with model antigen KLH, we assessed whether pNO2Phe-containing proteins could promote naïve CD4+ T cells to differentiate into Tfh cells with exposure to interleukin-12 (IL-12) and transforming growth factor-β (TGF-β). Consistent with previous reports, the proportion of CXCR5+PD-1+ Tfh cells was greater in the IL-12 or IL-12+ TGF-β condition than that in a cytokine-free condition (Figures 3a and 3b). The percentage of CXCR5+PD-1+ Tfh cells varied significantly at different concentrations of IL-12+ TGF-β (Figures 3a and 3b). DCs pulsed with these variant antigens improved the differentiation of IL-21-producing T cells (Figure 3c). What is more, pNO2Phe87hTNF-loaded DCs induced higher B cell lymphoma 6 (BCL-6) expression of T cells (Figure 3d). Along with IL-12+ TGF-β, these pNO2Phe-containing proteins-loaded DCs further increased BCL-6 and decreased Blimp-1 level (Figure 3d), which indicated a greater Tfh differentiation.

![Figure 1](image-url)  
*Figure 1.* Incorporation of pNO2Phe into surface-exposed sites of hTNF.  
**A.** X-ray crystal structure of hTNF- trimer with Tyr87, Lys11, pNO2Phe11 and pNO2Phe87 indicated (PDB ID code 1TNF). **B.** Expression of hTNF variants, as determined by SDS-PAGE. M: molecular weight marker. Lanes 1–3: pNO2Phe11hTNF. Lanes 4–6: pNO2Phe87hTNF. **C.** Purified hTNF, pNO2Phe11hTNF and pNO2Phe87hTNF (Lanes1-3) was assayed by 12% SDS-PAGE.

| Sample          | Fragment produced from trypsin digestion | Calculated mass (Da) | Observed mass (Da) |
|-----------------|------------------------------------------|----------------------|--------------------|
| hTNF            | TPSDYPVAHVNAPQAEGQLQWLNR                 | 2755.427             | 2755.435           |
| pNO2Phe11hTNF   | TPSDpNO2PFPVAHVNPQAEGQLQWLNR            | 2819.427             | 2819.396           |
| hTNF            | IAVSYQTK                                  | 909.504              | 909.505            |
| pNO2Phe87hTNF   | IAV5 pNO2PheFQTK                         | 938.505              | 938.494            |
Figure 2. Proliferation of naïve CD4^{+} T-cells after incubation with autologous antigens-pulsed DCs. CFSE-labeled naïve CD4^{+} T cells were co-cultured with DCs pulsed by autologous antigens and analyzed by flow cytometry. DCs were loaded with wild-type hTNF, pNO\textsubscript{2}Phe-containing hTNF and KLH.
Differentiation of specific antibody-secreting plasma cells by in vitro immunization

To investigate antigen-specific immune responses, we co-cultured in vitro-induced T cells with pre-activated autologous naïve B cells. Antibody titers cross-reacting with corresponding wild-type hTNF were determined by ELISA. CD4+ T cells primed by resting DCs, even under conditions of IL-12+ TGF-β, had no ability to induce secretion of specific antibodies (Figure 4a). CD4+ T cells primed by pNO2Phe-containing B-cell activating factor (BAFF)-loaded DCs also could not trigger secretion of anti-hTNF antibody. By contrast, activated Tfh cells, which were primed by pNO2Phe-containing hTNF-loaded DCs and IL-12+ TGF-β, helped naïve B cells to produce immunoglobulins (Igs) cross-reactive with wild-type proteins in vitro (Figure 4a). To determine the dynamics and magnitude of the human antibody response, we analyzed the frequency of antibody-secreting plasma cells in a time course manner after in vitro immunization. The anti-hTNF-secreting plasma cell response was transient, peaking at approximately day 12 and reduced at day 14 (Figures 4b and 4c).

Construction of antigen-specific antibody library and affinity selection

We succeeded in de novo generation of antigen-specific B cells by in vitro immunization, but the antigen-specific V\textsubscript{H} and V\textsubscript{L} genes could not be obtained. To overcome this problem, antibody-secreting plasma cells (CD19+/CD38+/CD3−) were sorted on day 12 for hTNF after in vitro immunization (Figure 5a). RT-PCR was performed using a cocktail of appropriate family-specific primers (Figure 5b). After ligation of V\textsubscript{H} and V\textsubscript{L} genes with phagemid vector, an antigen-specific antibody library was constructed by transforming E. coli TG1. Due to somatic hypermutation allowing for high-affinity antibodies, we investigated diversification at the Ig variable region by sequence analysis. Naïve B cells were also sorted for comparison. The in vitro immunized B cells against hTNF had significantly increased numbers of point mutations compared with that in naïve B cells (Figure 5c). As a result, high-affinity antibodies with hypermutation might be de novo produced in our in vitro immunization system.

To screen high-affinity antibodies, we chose native proteins as targets by the phage display method. It is important to effectively increase the phage clones interacting with the target by affinity panning. After three rounds of panning, we obtained numerous clones with high absorbance and antigen specificity from the antigen-specific antibody library (Figure 5d). We selected independent clones displaying high antigen binding specificity against hTNF for further studies.

Detection of antibody binding and in vitro neutralization activity

Our eukaryotic-expressed full-length IgG against hTNF was composed of proteins with an apparent molecular mass of 55 kDa and 26 kDa, which represented heavy chain and light chain (purity >90%) as analyzed by SDS-PAGE (Figure 6a). The results are shown through the Graphpad software analysis (Figure 6b), we found anti-hTNF antibody with antigen binding activity of EC\textsubscript{50} (1.7 nM). The result showed our antibody demonstrated effective TNF neutralization in L929 assay (Figure 6c).

Discussion

We previously described a co-culture system with naïve lymphocyte populations for de novo generation of a specific IgM response against KLH as a model antigen. Here, to further investigate whether our in vitro immunization system can be used on human autologous proteins, we established a more
general method that enables efficient production of specific IgGs.

In this study, we demonstrated that DCs pulsed with wild-type hTNF were unable to initiate naïve CD4⁺ T cell proliferation. This failure to induce T cell proliferation could be due to immune tolerance. In order to overcome self-tolerance, a number of approaches for in vitro immunization have been developed by different research groups. Borrebaeck et al.¹⁸ eliminated the immune suppressor cells with Leu-Leu-OMe before use. Fujiki et al.¹⁹ improved the tolerated antigens by a multiple antigen peptide for sensitization. Chin et al.²⁰ linked self-immunogens to a promiscuous CD4⁺ T epitope from tetanus toxin. However, these approaches were complicated and restricted to vaccinated donors having reactive T cells against neo-epitopes. Here, we used a genetic-code expansion system that allowed the expression of autologous proteins with site-specific incorporation of pNO₂Phe. Recently, Schultz et al.¹⁴ showed that the introduction of pNO₂Phe into an autologous protein offered a simple and effective approach to overcome self-tolerance in vivo. In accordance with this previous report, we observed that DCs loaded with these protein mutants could trigger naïve CD4⁺ T-cell activation in vitro. The results might be attributed to a strong CD4⁺ T cell epitope created by the introduction of pNO₂Phe, which is in agreement with the Chin et al. study.²⁰ Without the need for vaccinated donors, naturally occurring posttranslational modifications such as nitration resulted in a common CD4 repertoire for epitopes containing pNO₂Phe.²¹,²² In addition, we confirmed that the degree of naïve CD4⁺ T cell proliferation depended on sites incorporated with pNO₂Phe because not all sites corresponded to potential T cell epitopes.

Tfh cells are dedicated B cell helper T cells that initiate extrafollicular and germinal center antibody responses and are crucial for affinity maturation.²³–²⁵ Our previous studies suggested that Tfh cells were important for antibody generation in our in vitro immunization system. Here, we demonstrated that DCs pulsed with pNO₂Phe-containing self-antigens also contributed to the differentiation of human Tfh cells along with IL-12+ TGF-β. The Tfh phenotype (CXCR5, PD-1 and IL-21) was positively expressed. Exogenous IL-12+ TGF-β exhibited a strong effect on CD4⁺ T cells, leading to increased production of IL-21. The Tfh transcriptional signature (i.e., upregulation of BCL-6 and downregulation of Blimp-1) was strongly induced. Moreover, we also observed that the in vitro-induced Tfh cells had the capacity to help B cells. Indeed, Tfh cells, differentiated by pNO₂Phe⁸⁷ hTNF+, could raise naïve B cells to produce cross-reactive Igs against wild-type counterparts. The dynamics and magnitude of the human

Figure 4. Differentiation of specific antibody-secreting plasma cells by in vitro immunization.

A. Ig concentrations in the co-culture of activated T cells under different conditions and naïve B cells. Data from three independent experiments. B. Ig concentrations in the co-culture of in vitro induced Tfh cells and naïve B cells at different time points. Data from three independent experiments. C. Flow cytometry analysis of differentiation of specific antibody-secreting plasma cells at different time points. One-way ANOVA test. *p < 0.05 vs. group DC-pNO₂Phe⁸⁷ hTNF+ IL-12+ TGF-β. # p < 0.05 , ## p < 0.01. n.s., not significant.
antibody response was heavily dictated by the antigen types. At the peak time point, we sorted plasma cells by flow cytometry and an established antigen-specific antibody library. In addition, we demonstrated the diversity of Ig variable genes. Collectively, in vitro immunized by pNO₂Phe87hTNF, the sorted specific plasma cells had accumulated more VH gene mutations than naïve B cells. The current somatic hypermutation events agreed with our previous research in which Blimp-1 and Activation-induced cytidine deaminase (AID) was detected in our co-culture system. A variety of approaches have been devised for somatic hypermutation in vitro. Cumbers et al.26 generated affinity maturation of antibodies using hypermutating B-cell lines, and Seo et al.27 developed gene conversion in DT40 cells by treatment of a histone deacetylase inhibitor, trichostatin A. Compared with these established methods, our in vitro immunization system might be closer to the natural immune response, which was initiated at human naïve B cells.

During previous experiments, we only detected specific antibodies of the IgM class. Under class switching, the IgM are converted into IgG, which allows easier purification and more practical use. To mimic immune response in the germinatal center, we selected high-affinity Ig variable fragments by phage display and assembled them into IgGs. By phage display technology,28 the random pair of VH and VL region simulated the antibody affinity maturation in vivo, generating antibodies with high-affinity. Compared with conventional methods, construction of an antigen-specific antibody library in our system requires less time and fewer B cells. Consequently, we obtained human neutralizing IgG against hTNF.

We acknowledge that the antigen-specific B cell-response has obvious individual difference in our in vitro immunization system. In addition, sensitivity of naïve CD4⁺ T cells against antigen sensitization differs between donors. This is probably due to the great variety of human genetic background. Schultz et al.15 found that introduction of pNO₂Phe at a particular site in self-antigens breaks tolerance in the context of a specific MHC. Moreover, Holmdahl et al.16 reported pNO₂Phe-containing variants showed different immunogenicity in mice on different MHC II

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**Figure 5.** Construction of antigen-specific antibody library and affinity selection.

**A.** Antibody-secreting plasma cells were sorted directly into Trizol for RNA extraction by flow cytometry. **B.** Amplification of VH and VL by RT-PCR with specific primers. **C.** The percent of all variable genes from B-cell population with the number of somatic mutations denoted in the legend. VH variable genes of in vitro immunized B cells were compared with germline genes. VH variable genes of naïve B cells were analyzed as negative control. **D.** After 3 rounds of panning, hTNF-specific phage antibody was respectively selected from separate in vitro immunized specific antibody library. BSA was used as the non-specific and negative-control antigen.
backgrounds. To clarify this issue, we plan to investigate more thoroughly the human MHC II locus restricted to pNO2Phe-containing self-antigens, leading to a more consistent and efficient antigen-specific B-cell response in our system. We can also introduce pNO2Phe into potential T cell epitopes through the analysis of bioinformatics software. Further, we can use genetic incorporation of pNO2Phe into more sites on a self-protein, creating a higher potency antigen with more universal immunogenicity.

In summary, we established a more general in vitro immunization method to produce specific IgGs against human autologous proteins. Our co-culture system is initiated with exceedingly low affinity naïve B cells, but antibodies with nanomolar affinities can be obtained. Through incorporation of unnatural amino acids into natural proteins, this new system provides more options for generating high-affinity mAb therapeutics against targets of self-proteins.

Materials and methods

Plasmid construction

The coding sequence of human TNF (NCBI accession no. AAC03542.1) was synthesized by GenScript. The hTNF gene was digested with Ncol and BamHI restriction enzymes (Takara, 107824–63-5 and 81295–09-2) and subsequently cloned into a pET-28a vector. His6-tag was introduced into these two vectors at the 5’ end of the gene sequences. Site-specific incorporation of pNO2Phe into hTNF was carried out by mutating the codon for Tyr87 or Lys11, to a TAG amber codon by using the Fast Mutagenesis Kit (Vazyme, C215-01/02) (site previous publication on UAA incorporation methodology).14 These mutant proteins were designated as pNO2Phe87hTNF and pNO2Phe11hTNF, respectively. The sequences of all constructs were confirmed by DNA sequencing.

Purification and characterization of proteins

To express pNO2Phe-containing variants, these mutant plasmids were separately transformed into E. coli BL21 (DE3) cells with a plasmid containing Methanococcus jannaschii–derived aminoacyl–transfer RNA synthetase/transfer RNA pair specific for pNO2Phe. The recombinant strains were cultured in GMML medium containing 50 μg/ml kanamycin, 25 μg/ml chloramphenicol, and 4 mM pNO2Phe, and the cells were grown at 37°C and 220 rpm until OD600 reached to 0.8–1.0. The cultures were further incubated with 1 mM isopropyl-β-d-thiogalactoside (BioSharp, BL545A), and cells were grown at 37°C for additional 18–20 h. Wild-type proteins were expressed in LB medium in the absence of pNO2Phe. The cells were pelleted, and the protein was purified by Q Sepharose Fast Flow column and Ni2+ affinity chromatography according to the manufacturer’s instructions (GE Healthcare, 17051010 and 17526801). Endotoxin was removed with Endotoxin Affinity Resin (Thermo Scientific, 88276) and proteins were shown to have <0.2 EU/ml endotoxin by limulus test agents (Chinese materials research center, 150603). The purity of protein was determined by SDS-PAGE. The detection of human TNF was identified by Western blotting with Rabbit Anti-Human TNF-α (PeproTech, 96–500-P31A-100). Successful incorporation of pNO2Phe into mutant proteins was verified by tryptic in-gel digestion and subsequent MS/MS fragmentation on a Xevo-G2-XS-QTOF mass spectrometer (Waters).

Isolation of human peripheral blood mononuclear cells

This study was performed in accordance with the principles of Declaration of Helsinki and was approved by Jiangsu Provincial Department for Health (Nanjing, China). Human peripheral blood Buffy coats from healthy donors, screened negative for HIV-1/2, HTLV-1/II, HCV, HBsAg, were obtained from Jiangsu Province Blood Center (Nanjing, China). They were collected from volunteers after signing of informed consents for the collection of blood apheresis samples. Peripheral blood mononuclear cells (PBMCs) were harvested by density-gradient centrifugations with Lymphocyte® (Cedarlane Laboratories, CL5026). Cells were washed three times with AIM-V medium (Gibco, 12055091).

Stimulation of naïve CD4+ T cells by self-antigen-loaded DCs and different cytokine cocktails in vitro

Monocyte-derived DCs were generated as described previously. Briefly, PBMCs were seeded onto 6-well culture plates...
(Costar, 3516) for two hours. After discarding cell suspension, DCs were generated by culturing adherent monocytes in AIM-V medium containing 500 U/ml IL-4 and 800 U/ml granulocyte-macrophage colony-stimulating factor (R&D, 204-IL-050 and 215-GM-050) for 7 days. At day 4, hTNF, pNOS2Phe\(^2\)hTNF or pNOS2Phe\(^3\)hTNF was respectively added as an autologous antigen. After 24 h stimulation with TNF-α (R&D, 210-TA-020), DCs were harvested and carefully washed. Autologous naïve CD4\(^+\) T cells (purity >95%, viability >90%) sorted by the human naïve CD4 \(+\) T Cell Isolation Kit II (Miltenyi, 130-091-150) were stained with anti-CD4 FITC (RPAT4), anti-CXCR5 PerCP-Cy\(^5.5\) (RF8B2) and anti-CD19 PE-Cy\(^7\) (HIB19) and anti-PD-1 APC (MIH4) mAbs. Expression of IL-21 was detected by intracellular staining. The phenotypes and the cytokine expression profiles were analyzed by flow cytometry. Expression of BCL-6 and Blimp-1 was determined by Western blotting.

**In vitro immunization by co-cultures of T-B cells**

Naïve CD4\(^+\) T cells were also differentiated by autologous antigen loaded DCs and different concentrations of IL-12+ TGF-β (R&D, 219-IL-025 and 240-B-010). Activated T cells were stained with anti-CD4 FITC (RPAT4), anti-CXCR5 PerCP-Cy\(^5.5\) (RF8B2) and anti-CD19 PE-Cy\(^7\) (HIB19) and anti-CD3 PE-Cy\(^7\) (HIB70) mAbs. Expression of IL-21 was detected by intracellular staining. The phenotype and the cytokine expression profiles were analyzed by flow cytometry. Expression of BCL-6 and Blimp-1 was measured by Western blotting.

**Generation of antigen-specific antibody library**

Antibody-secreting plasma cells (CD19\(^+\)/CD38\(^+\)/CD3\(^-\)) were sorted from our *in vitro* immunization system by fluorescence-activated cell sorting (BD FACS Aria, USA). Variable regions of the heavy chain genes (V\(_H\)) and light chain genes (V\(_L\), including V\(_\kappa\) and V\(_\lambda\)) from sorted B cells were amplified in a one-step RT-PCR reaction (TransGen Biotech, China) using a cocktail of appropriate family-specific primers. VH and VL were amplified by 30 PCR cycles at 94°C 30 s, 55°C 30 s, and 72°C 1 min. PCR products were then sequenced. After incorporating restriction sites at the ends of the variable genes, V\(_H\) and V\(_L\) genes were connected by the DNA linker and inserted into the phagemid vector pCANTAB-5E with T4 ligase (NEB, #M0202L). The ligated product was transformed by electroporation into *E. coli* TG1. The transformed cells were grown at 37°C in 2× YT medium supplemented with 100 μg/ml ampicillin and 2% glucose. The antibody library specific for hTNF was established by *in vitro* immunization with pNOS2Phe\(^2\)hTNF.

**Analysis of somatic mutations of variable region genes**

For analysis of somatic hypermutation frequency, V\(_H\) gene libraries after sequencing were considered. Background mutation rates were determined by analyses of a portion of the FACS-sorted naïve B cells with germline gene repertoires that is not subjected to somatic hypermutation. Through IgBLAST software, sequences of FACS-sorted antibody-secreting plasma cells by *in vitro* immunization were aligned to the parental clone of germline gene to determine the frequency of point mutation events.

**Affinity panning and phage ELISA**

Phage panning to wild-type hTNF immobilized to Maxisorp\(^∗\) Immunotubes (Nunc, 442402) was performed essentially as described. Briefly, phage antibody library specific for hTNF were incubated with coated hTNF for 2 h at room temperature. The bound phages were eluted with 100 mM TEA (Sigma, 471283) and used to infect *E. coli* TG1 cells. The infected cells were rescued by the addition of the helper phage and used for the next round of selection. After three rounds of selections, phage antibodies were obtained and tested for binding to hTNF.

To analyze the antigen specificity of phage antibodies, microtiter plates (Abi, GEN601079) were coated with hTNF. BSA (BioFROXX, 9048–46-8) was also coated as negative control. Phage antibodies in blocking buffer was added to each well and incubated for 1 h at 37°C. The plates were washed, and the bound phage was detected by an anti-M13 HRP-conjugated secondary antibody (Sino Biological,11973-MM05-B-50). The immunoreaction was developed by incubation with TMB liquid substrate (Solarbio, PR1210) and stopped by the 2 M H\(_2\)SO\(_4\). The results were measured at 450 nm by a microplate reader.

**Construction and purification of recombinant human monoclonal IgG**

Human IgG1 antibodies were constructed by cloning the V\(_H\) and V\(_L\) chain variable regions into expression vectors containing human IgG, or Igκ constant regions. Plasmids containing the heavy and light chain Ig genes were then co-transfected into the CHO cell lines by using Lipofectamine (Invitrogen, 11668027). Human IgG1 mAbs were expressed and purified using Hitrap\(^∗\) Protein A HP (GE Healthcare, 1704203).

**Indirect ELISA assay**

Indirect ELISA was used to determine the binding activity of antibody and antigen. Microtiter plates were coated for 16 h at 4°C with 1 μg/well wild-type hTNF diluted in carbonate sodium buffer (pH 9.6). The plates were blocked for 2 h at 37°C with 3% BSA dissolved in phosphate-buffered saline (PBS). Three-times diluted antibodies were added to each well and HRP-conjugated goat anti-human IgG secondary antibody was used for
In vitro neutralization assay

HTNF causes cell cytotoxicity to L929 cells after an incubation period of 18–24 h. The ability of anti-hTNF antibody to neutralize HTNF activity was determined after incubating antibody with hTNF. L929 cells were plated on 96-well plates with different concentrations of expressed antibody and 1 pg/ml hTNF. L929 cells were cultured for 18 h in the presence of actinomycin D (1 µg/ml). MTT was added to detect cell activity.

Statistical analysis

Statistical evaluation of the data was conducted using SPSS Version 19.0. Shapiro-Wilk tests were used to determine whether the dataset (3 ≤ n < 50) belongs to a normal distribution population. The significance of the difference between groups was analyzed with One-way ANOVA test with Bonferroni correction. Differences were considered statistically significant at *p < 0.05, **p < 0.01 and ***p < 0.001.

Abbreviations

MAbs monoclonal antibodies
TNF tumor necrosis factor
KLH Keyhole limpet hemocyanin
BAFF B-cell activating factor
PBMCs Peripheral blood mononuclear cells
Tfh Follicular helper T
DCs Dendritic cells
BCL-6 B cell lymphoma 6
AID Activation-induced cytidine deaminase

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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