

IL17A Autoantibody Induced by Recombinant Protein Ag85A-IL17A

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Abstract IL17A is a widely studied cytokine which plays an important role in autoimmune diseases as well as asthma. In this study, murine IL17a gene fragment was inserted into pET28a-Ag85A and it was expressed as a fusion protein named Ag85A-IL17A. Recombinant protein Ag85A-IL17A was expressed as an open reading frame of 1,287 bp, encoding a polypeptide of 429 amino acid residues with a predicted molecular mass of 50 kDa. It was expressed in *Escherichia coli* BL21 and purified by metal affinity chromatography using a nickel–nitrilotriacetic acid column. Bioinformatics revealed that Ag85A-IL17A contained B cell epitopes of IL17A. Purified proteins Ag85A-IL17A and Ag85A were injected with adjuvants into mice then antibody responses in sera were measured by enzyme-linked immunosorbent assay and western blot assay. Experiments showed that purified protein Ag85A-IL17A could bind to a standard IL17A antibody and could induce IL17A autoantibody in mice.

Keywords IL17A · Ag85A · Cloning · Expression · Autoantibody · B cell epitope

Introduction

Asthma has long been considered as a chronic airway inflammation of dysregulated T helper type 2 (Th2) immune responses to environmental allergens [1]. Certain antibodies such as IL4 antibody and IL13 antibody show therapeutic effect or prophylaxis effect in murine asthmatic model [2, 3]. Emerging evidences suggest that IL17A contributes to the development of asthma, especially in severe asthma which has a characteristic neutrophil infiltration in the airways [4–6]. Patients with severe asthma have a higher level of IL17A in induced sputum compared to healthy people [7–9]. Thus antibody to IL17A might be beneficial in neutralizing IL17A in severe asthma. However, it has shown that administration of an IL17A-blocking antibody could escalate Th2 cytokines such as IL13 in experiments in mice [10]. Epidemiological assessment of patients and animal models suggest that exposure to *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) vaccine in early life might prevent

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asthma development [11]. BCG vaccine stimulates Th1 responses with increased IFN- γ and IL-12, which in turn inhibits cytokines from Th2-like cells as well as proliferation of Th2-like cells [12]. BCG immunization can also suppress preexisting Th2-type immune response in atopic children with asthma [13]. The specificity and mechanisms of these effects are not clear but the mycobacterial antigen Ag85A is one of a family of three immunodominant protein antigens in BCG that might have a role [14–16].

Under normal conditions, self-reactivity is prevented by a number of processes resulting in immunological tolerance of self-antigens. Therefore, cytokines do not normally induce autoantibodies in vivo. Boissier et al. speculated that when a self-protein is associated with a foreign protein carrier it could induce B cell responses and autoantibodies via Th epitopes of foreign protein resulting in neutralization of the self-protein [17]. Therefore, we decided to construct a recombinant protein using Ag85A as a protein carrier to provide Th epitopes to induce murine IL17A autoantibody. The recombinant protein Ag85A-IL17A was expressed, purified, and identified for its immunogenicity and antigenicity which laid the foundation for further study.

Materials and Methods

Construction and Expression of Recombinant Protein Ag85A-IL17A

The plasmids pET28a-Ag85a and pET28a-IL17a were constructed by our laboratory. Murine IL17a (NM_010552.3) was identified and amplified by PCR using the following primers with Acc I restrictive endonuclease sites and PCR conditions: forward, 5'- CCG GTCTAC GCA GCG ATC ATC CCT -3', reverse, 5'- CG GTAGAC GGC CTG CTG GAC -3'; 94 °C for 30 s, 56 °C for 40 s, 72 °C for 30 s for 30 cycles, followed by extension for 6 min at 72 °C. The primers were synthesized by Invitrogen Trading Co. (Shanghai, China). The PCR product and pET28a-Ag85a were digested by restrictive endonuclease Acc I (Fermentas) at 37 °C for 2 h [18]. Then, the PCR product was cloned into pET28a-Ag85a via T4 DNA ligase (Fermentas) and transformed to competent *Escherichia coli* DH5 α [19]. Transformants were identified by endonuclease digestion and sequencing. The confirmed plasmid with correct sequence was transformed into competent *E. coli* BL21(DE3) for protein expression. Three clones were incubated into 5 ml fresh Luria–Bertani broth medium containing 50 μ g/ml kanamycin. The medium was cultured till optical density (OD) reached 0.6 at 600 nm and then induced by isopropyl- β -D-thiogalactoside (IPTG; Sango, Shanghai, China) at a final concentration of 1 mM for 4 h at 37 °C [20].

Purification, Denaturation, and Renaturation of Recombinant Protein Ag85A-IL17A, Ag85A, and IL17A

Two hundred milliliters of *E. coli* BL21(DE3) cells containing pET28a-Ag85a-IL17a was cultured till OD reached 0.6 at 600 nm and then were induced by IPTG at a final concentration of 1 mM for 4 h at 37 °C. The resultant medium was centrifuged (4,000 $\times$ g, 10 min at 4 °C) and the pellet was re-suspended into 20 ml of phosphate-buffered saline (PBS). The cells were broken by ultrasound with 100 W for 20 min then centrifuged (12,000 $\times$ g, 10 min at 4 °C) and the supernatant was discarded. The sediment was re-suspended in buffer A (20 mM Tris-Cl, 500 mM NaCl, 8 M urea) and incubated at 4 °C overnight. The supernatant was loaded onto pre-equilibrated nickel–nitrilotriacetic acid agarose (QIAGEN) [21]. After that, the resin was rinsed by buffer B (20 mM Tris-Cl,

500 mM NaCl, 8 M urea, and 5 mM imidazole) and at last the binding protein was eluted by a buffer C (20 mM Tris-Cl, 500 mM NaCl, 8 M urea, and 150 mM imidazole). The protein was then dialyzed in TGE dialysis medium (50 mM Tris, 0.5 mM EDTA, 50 mM NaCl, 10 % glycerol, pH7.0) with concentrations of urea at 8, 6, 4, 2, and 0 M. Following this removal of urea from solution, the recombinant protein Ag85A-IL17A had refolded. The recombinant proteins Ag85A and IL17A were obtained following the same protocol. The purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue R-250 staining [22]. The concentrations of purified proteins were determined by the Bradford method with serum from bovine albumin (BSA) as a standard [23].

Prediction of B Cell Epitopes and 3D Structure of Recombinant Proteins

Potential B cell epitopes of recombinant proteins Ag85A-IL17A and IL17A were identified using epitope prediction software online (<http://www.imtech.res.in/raghava/bcepred/>) [24]. For the 3D structure of recombinant protein Ag85A-IL17A, the protein amino acid sequence was sent to Swiss-Model Workspace to get a PDB file and then PyMol CPH software was applied to display the 3D structure of recombinant protein Ag85A-IL17A [25].

Immunogenicity of Recombinant Protein Ag85A-IL17A

IL17A capture antibody (eBioscience, 100 µl/well) was coated on 96-well microtiter plate at 4 °C overnight. After washing five times with PBST (PBS, 0.5 % Tween 20), the plate was blocked with 1× Assay Diluent (eBioscience) for 1 h at room temperature. After triplicate washing with PBST, the wells were incubated with 100 µl standard protein of IL17A (eBioscience), recombinant protein IL17A, recombinant protein Ag85A, or recombinant protein Ag85A-IL17A at a range of concentrations at room temperature for 2 h. The wells were then washed with PBST six times, and 100 µl/well IL17A detection antibody (eBioscience) was added and incubated in the plate at room temperature for 1 h. After washing the plate with PBST six times, Avidin-HRP (eBioscience) was added and incubated in the plate at room temperature for 30 min. Substrate solution (eBioscience) was added to wells and the plate was incubated for 3 min at room temperature and finally the reactions were stopped by adding 2 M H₂SO₄ to the wells.

Animal Immunization

Female Balb/c mice, 4–6 weeks of age were purchased from Shanghai Public Health Clinical Center and raised in Specific Pathogen Free laboratory animal facility of the center. They were randomly divided into three groups of five animals: Ag85A-vaccinated group, Ag85A-IL17A-vaccinated group, and PBS group. Vaccinated groups were injected subcutaneously with 400 µl of 1:1 emulsion of 50 µg recombinant protein in complete Freund's adjuvant (Sigma) per mouse. Two, 5, and 8 weeks later, vaccinated groups received intraperitoneal injection of 50 µg recombinant protein in incomplete Freund's adjuvant (Sigma). Control mice were injected with 400 µl PBS each time. All mice were sacrificed 2 weeks after the last injection and sera were collected.

Antigenicity of Recombinant Protein Ag85A-IL17A

The concentration of IgG antibody against IL17A in sera from each mouse was measured by enzyme-linked immunosorbent assay (ELISA). Purified recombinant protein IL17A at a

concentration of 2.5 µg/ml in carbonate/bicarbonate buffer (pH9.6) was coated on 96-well microtiter plate and incubated at 4 °C overnight. After washing with PBST five times, the wells were blocked with 2 % BSA at room temperature for 2 h. After triplicate washing with PBST, wells were incubated with sera at room temperature for 1 h. Sera were serially diluted in 2 % BSA to 1:200, 1:400, 1:800, 1:1,600, and 1:3,200. Goat anti-mouse IgG-HRP (KPL; 1:6,000 diluted in 2 % BSA) was added and wells were incubated at room temperature for 30 min. Substrate solution (eBioscience) was then added to wells and incubated for 3 min and reactions were stopped with 2 M H₂SO₄. Data are presented as mean of OD value at 450 nm per group.

Western Blot Analysis

Western blot analysis was carried out as described previously [26]. Briefly, purified recombinant proteins were subjected to electrophoresis on 12 % SDS-PAGE. Then, proteins separated on gel were transferred onto PVDF membranes (Millipore) and the membranes were blocked in PBS with 5 % nonfat dry milk at room temperature for 2 h. Blots were then incubated at 4 °C overnight in sera from mice vaccinated with different proteins (diluted 1:2,000). After five washes with PBST, blots were incubated for 1 h in HRP-conjugated goat anti-mouse IgG antibody (KPL; 1:6,000 diluted with PBST). The reaction was detected by Western Lighting Plus-ECL (Perkin Elmer).

Results and Discussion

Construction and Expression of Recombinant Protein Ag85a-IL17a

The IL17a gene fragment amplified from pET28a-IL17a and digested by Acc I restrictive endonuclease was successfully subcloned into pET28a-Ag85a digested by Acc I restrictive endonuclease. The pET28a-Ag85a-IL17a construct was identified by analysis of endonuclease digestion as shown in Fig. 1a. The fragment of pET28a-Ag85a that was obtained by Nco I and Xho I had a mobility consistent with a size of 800 bp as expected. Similarly, the fragment of IL17a gene that was inserted into pET28a-Ag85a had the expected size of about 400 bp and the fragment of pET28a-Ag85a-IL17a after digestion by Nco I and Xho I had about 1,200 bp. Digestion of pET28a-Ag85a-IL17a by Acc I yielded a fragment of about 400 bp, indicating that the fragment of IL17a gene was successfully inserted into pET28a-Ag85a. After expression in *E. coli* BL21(DE3) cells, the recombinant proteins IL17A, Ag85A, and Ag85A-IL17A were purified and analyzed by SDS-PAGE. As Fig. 1b showed, recombinant protein IL17A had an apparent mass of about 17 kDa, recombinant protein Ag85A was about 35 kDa, and recombinant protein Ag85A-IL17A was about 50 kDa.

Prediction of B Cell Epitopes of Recombinant Protein Ag85A-IL17A

Online epitope prediction software indicated that recombinant protein Ag85A-IL17A had three B cell epitopes of IL17A: LGAKVSSRRPSDYLN₁₇₆₋₁₉₃, TLHRNEDP₁₉₇₋₂₀₄, and LVLKREP₂₄₂₋₂₄₈. To visualize the 3D structure of recombinant protein Ag85A-IL17A, amino acid sequence of protein was sent to Swiss-Model Workspace to get a PDB file and then PyMol CPH software was applied to display the 3D structure. The three epitopes on Ag85A-IL17A that corresponded to those of IL17A were respectively shown in red, blue, and green (Fig. 2). Since recombinant protein Ag85A-IL17A displayed the B cell epitopes of IL17A and had Th epitopes of Ag85A, we inferred that it might induce IL17A autoantibody in vivo.

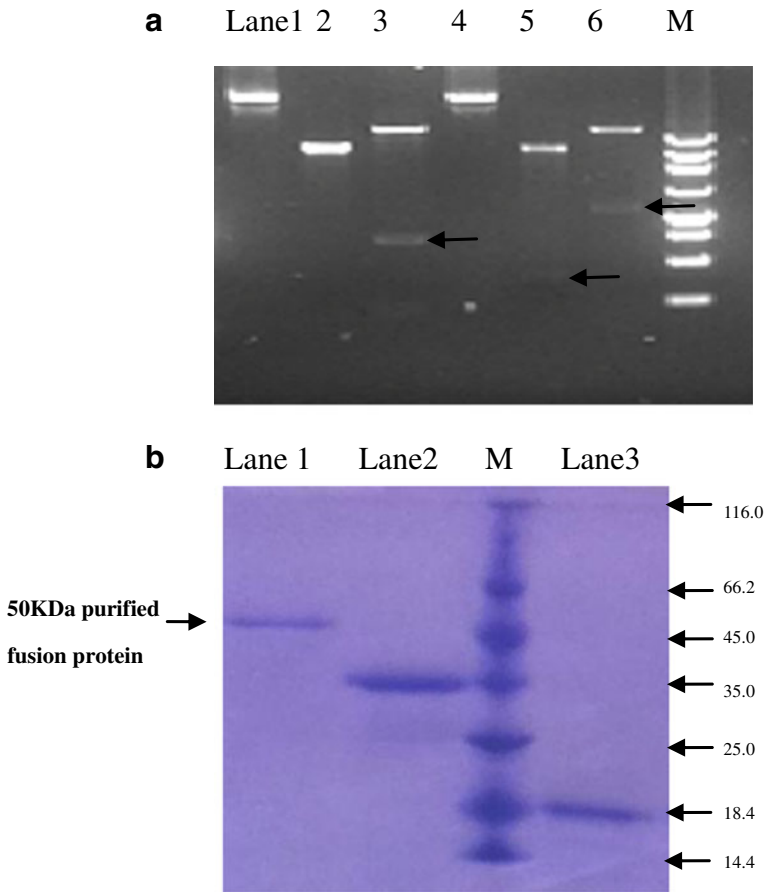
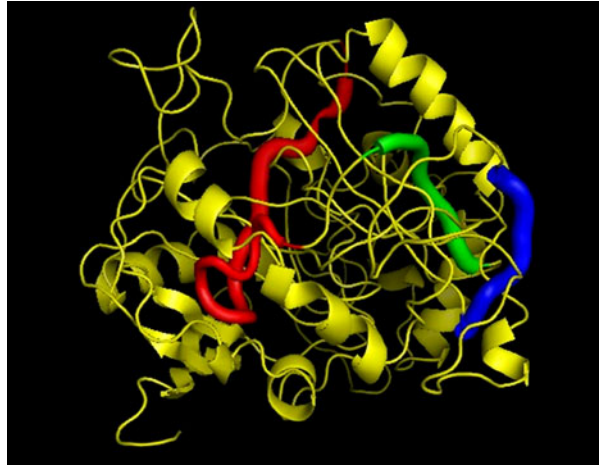


Fig. 1 Construction of pET28a-Ag85a-IL17a and expression of recombinant protein Ag85A-IL17A. **a** pET28a-Ag85a-IL17a was identified by the analysis of endonuclease digestion. *Lane M* shows 250 bp DNA ladder marker (Takara) with fragment sizes 4,500, 3,000, 2,250, 1,500, 1,000, 750, 500, and 250 bp. *Lane 1* and *lane 4* show pET28a-Ag85a and pET28a-Ag85a-IL17a without digestion. *Lane 2*, pET28a-Ag85a digested by *Acc I*. *Lane 3*, pET28a-Ag85a digested by *Nco I* and *Xho I*. *Lane 5*, pET28a-Ag85a-IL17a digested by *Acc I*. *Lane 6*, pET28a-Ag85a-IL17a digested by *Nco I* and *Xho I*. **b** Purified recombinant proteins Ag85A-IL17A, Ag85A, and IL17A were analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue R-250 staining. *Lane M* contains molecular weight markers (Fermentas) with the masses shown, in kilodalton, to the right with arrows. *Lane 1*, purified recombinant protein Ag85A-IL17A. *Lane 2*, purified recombinant protein Ag85A. *Lane 3*, purified recombinant protein IL17A

Immunogenicity of Recombinant Protein Ag85A-IL17A Revealed by ELISA

As a first test of the likely immunogenicity of recombinant protein Ag85A-IL17A, binding ability to specific antibody was tested. Commercial IL17A capture antibody (eBioscience) was coated on a 96-well plate at 4 °C overnight. Recombinant proteins Ag85A-IL17A, IL17A, and Ag85A and standard protein IL17A were added and allowed to bind for 2 h at room temperature. The initial concentration of each protein was 250 µg/ml and was serially diluted to 1/30, 1/60, 1/120, and 1/240 with 2 % BSA. As shown in Fig. 3, the binding ability of recombinant protein IL17A to IL17A capture antibody was similar to standard protein IL17A. Recombinant protein Ag85A-IL17A could also bind to IL17A capture antibody while recombinant protein

Fig. 2 3D structure and B cell epitopes of recombinant protein Ag85A-IL17A. Three B cell epitopes LGAKVSSRRPSDYLNRS₁₇₆₋₁₉₃, TLHRNEDP₁₉₇₋₂₀₄, and LVLKREP₂₄₂₋₂₄₈ are shown in *red*, *blue*, and *green* in 3D structure of recombinant protein Ag85A-IL17A



Ag85A did not bind. Thus, recombinant protein Ag85A-IL17A had affinity to IL17A antibody. The B cell epitopes shown in Fig. 2 would account for this phenomenon. It also implied that recombinant protein IL17A might have similar immunogenicity compared to standard protein IL17A and could be used as coating antigen in subsequent experiments.

Antigenicity of Recombinant Protein Ag85A-IL17A Revealed by ELISA

Since recombinant protein IL17A had almost the same binding ability compared to standard protein IL17A, we used the recombinant protein IL17A as coating antigen to detect the antibody of IL17A in the sera of vaccinated mice. Recombinant protein IL17A was coated in 96-well microtiter plates at a concentration of 2.5 µg/ml in carbonate/bicarbonate buffer (pH 9.6). Sera from variously vaccinated mice were used as first antibody and goat anti-mouse IgG-HRP was used as second antibody. As shown in Fig. 4, recombinant protein Ag85A- and PBS-immunized mice had no IL17A-specific IgG in sera. Recombinant protein Ag85A-IL17A-immunized mice had high titers of IgG specific to IL17A in sera. Thus, recombinant protein Ag85A-IL17A could induce IL17A-specific IgG *in vivo*. The presence of antibody to IL17A in the sera of mice vaccinated with Ag85A-IL17A was consistent with the presence

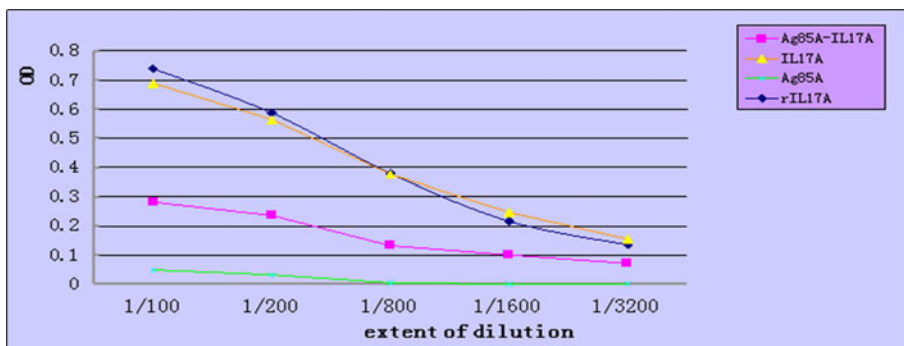


Fig. 3 Immunogenicity of recombinant protein Ag85A-IL17A revealed by ELISA. OD 450 nm showing the binding ability of recombinant proteins IL17A, Ag85A, and Ag85A-IL17A as well as standard protein IL17A to IL17A capture antibody. Each point represents the average of three independent experiments

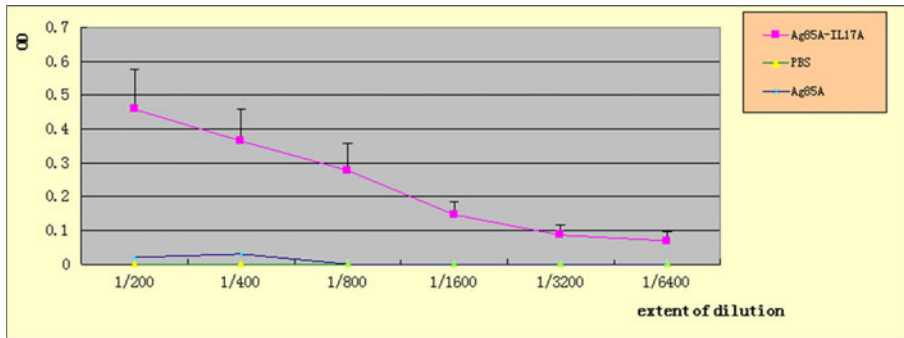


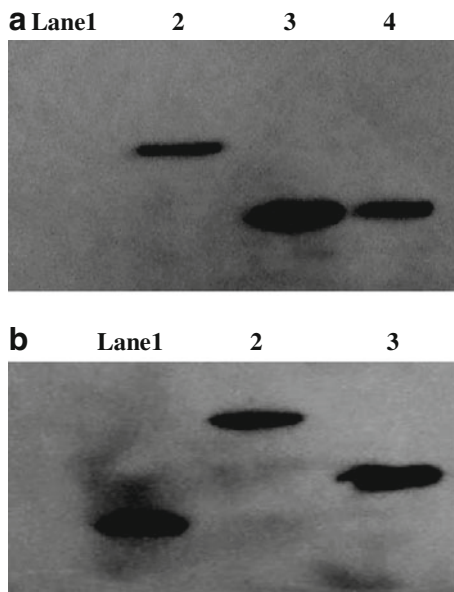
Fig. 4 Antigenicity of recombinant protein Ag85A-IL17A by ELISA. OD 450 nm showing concentrations of antibody specific to IL17A in sera from mice vaccinated with recombinant protein Ag85A, recombinant protein Ag85A-IL17A, and PBS. Each group contained five mice. Vaccinated groups were injected subcutaneously with 400 μ l of 1:1 emulsion of 50 μ g recombinant protein in complete Freund's adjuvant. Two, 5, and 8 weeks later, vaccinated group received intraperitoneal injection of 50 μ g recombinant protein in incomplete Freund's adjuvant. Control mice were injected with 400 μ l PBS each time. All mice were sacrificed 2 weeks after the last injection to provide serum

of three B cell epitopes of IL17A (Fig. 2) and foreign Th epitopes, and confirmed the speculation of Boissier et al. that foreign protein carrier could induce B cell responses and generate autoantibodies via Th epitopes of the foreign protein.

Immunogenicity and Antigenicity of Recombinant Protein Ag85A-IL17A Revealed by Western Blot

To further study the immunogenicity and antigenicity of recombinant protein Ag85A-IL17A, recombinant proteins IL17A, Ag85A, and Ag85A-IL17A were transferred to PVDF

Fig. 5 Immunogenicity and antigenicity of recombinant protein Ag85A-IL17A revealed by Western blot. Purified recombinant proteins IL17A, Ag85A, and Ag85A-IL17A were transferred from SDS-PAGE to PVDF membrane. Sera containing IL17A-specific antibody bound to the protein on PVDF membranes and was identified by HRP-conjugated goat anti-mouse IgG antibody. **a** Serum from recombinant Ag85A vaccinated mice was used as first antibody. Lane 1, recombinant protein IL17A. Lane 2, recombinant protein Ag85A-IL17A. Lanes 3 and 4, recombinant protein Ag85A. **b** Sera from recombinant protein Ag85A-IL17A-vaccinated mice was used as first antibody. Lane 1, recombinant protein IL17A. Lane 2, recombinant protein Ag85A-IL17A. Lane 3, recombinant protein Ag85A



membrane. Sera from variously immunized mice was diluted to 1:2,000 and used as first antibody in western blotting. As shown in Fig. 5a, sera from mice vaccinated with recombinant protein Ag85A had antibody binding to recombinant protein Ag85A and Ag85A-IL17A but not to recombinant protein IL17A. Figure 5b showed that sera from recombinant protein Ag85A-IL17A-immunized mice had antibody which could bind to recombinant protein IL17A, Ag85A, and Ag85A-IL17A. As recombinant protein Ag85A did not induce antibody to bind protein IL17A while recombinant protein Ag85A-IL17A did, the detected antibody in sera of mice vaccinated with Ag85A-IL17A was antibody against IL17A. This confirmed that Ag85A-IL17A had the same B cell epitopes as IL17A and it might induce IL17A autoantibody in vivo. Therefore, because studies have shown patients with severe asthma have a higher level of IL17A in induced sputum compared to healthy people, IL17-specific antibody induced by this recombinant protein might have a beneficial effect in neutralizing IL17A cytokine in such people.

The actual mechanisms underlying such breaking of tolerance to induce autoantibody are not known. However, it might be that B cells binding to the foreign protein have the potential to process and present the foreign component to T cells that are reactive to the foreign epitopes. Thus it is possible for T cells to subsequently deliver helper signals to B cells binding to the self-component, stimulating the production of autoantibody in vivo [27]. In our murine model, recombinant protein Ag85A-IL17A may be recognized by B cells because of Th epitope of Ag85A. Then T cells reacting to the Th epitopes deliver helper signals to B cells which stimulates the production of autoantibody to IL17A.

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