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Development and Characterization of Monoclonal Antibodies Against Mouse TSLP

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Epithelial-derived thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine that is mainly produced by epithelial cells. It has been shown to play a key role in the development of Th2-type allergic inflammation. Commercial antibodies available against TSLP can only be used in Western blot assay, which further limits investigation of its function. Here we efficiently generated a panel of anti-mouse TSLP monoclonal antibodies in rats with DNA priming-protein boosting strategy. Overall, four MAb strains (4B12, 4E11, 5D7, and 6H10) were obtained and their characterizations were identified. The MAbs can specifically bind to TSLP according to the ELISA and FACS assays. It was found that they recognized distinct epitopes. They are useful in detecting TSLP expression in the tissue by immunoblotting and in the cytoplasm by intracellular staining assay. Thus, these antibodies will be valuable tools for studying TSLP biological functions.

Introduction

Thymic stromal lymphopoietin (TSLP) was first identified from the conditioned medium of a mouse thymic stromal line that supported the development of B cells. The activities of mouse TSLP (mTSLP) overlap with those of IL-7.[1] Both mouse and human TSLP are expressed predominantly by epithelial cells, mostly in the lung, skin, and gut.[2] Compelling evidence was recently provided that TSLP may have a determinant role in the initiation and maintenance of the allergic immune response.[3,4] TSLP was initially shown to activate and instruct human $CD11c^+$ DCs to promote the differentiation of naive $CD4^+$ T cells into Th2 pro-inflammatory effectors, defined by the production of high levels of pro-allergic cytokines IL-4, IL-5, IL-13, TNF, and low levels of IL-10.[5,6] The role of TSLP in allergic diseases was subsequently supported by findings that it was specifically overexpressed in the acute and chronic lesions of atopic dermatitis (AD) patients and in the bronchi of asthmatic patients, where its expression level correlated with the severity of the disease.[3,7] The ability of TSLP to act as the initiating cytokine at the top of a chain of immunological events that lead to the atopic syndrome has been formally demonstrated in animal models.[8–10] Over-expression of the TSLP gene specifically in airway epithelial cells or keratinocytes led to asthma- and AD-like diseases, respectively.[9,10]

However, the biological function of TSLP has not been fully understood. Most of the commercial antibodies against TSLP can only be used in Western blot assay, which is an obstacle to experimental investigations about TSLP. In this study, we tried to develop a panel of monoclonal antibodies against mouse TSLP to provide tools for studying TSLP biological functions.

Materials and Methods

Construction of expression vectors

The full-length cDNA sequence of TSLP was amplified from the thymus of a BALB/c mouse by RT-PCR method. The primers (forward: 5′-CGTCGTGAATTCATGGTTCTTCTC AGGAGC-3′; reverse: 5′-GCTAGGCTCGAGCATAGCAGA AGCTGAAAG-3′) containing the synthetic *EcoR*I/*Xho*I sites were designed according to the published sequence (Genbank no. AF232937). The product was cloned into the *EcoR*I/*Xho*I (TaKaRa, Dalian, China) sites of pcDNA3.0 (Invitrogen, Carlsbad, CA) vector. The full length was also re-amplified from the template mTSLP/pcDNA3.0 by PCR method. The forward primer was the same as above. The reverse primer containing synthetic *BamH*I was as follows: 5′-ACGTATG GATCCCCCGATCCACCTCCGCCAG-3′. The PCR product was cloned into the modified pEGFP-N1 vector (modified in our laboratory), in which an ~20 aa transmembrane fragment was inserted into the upstream of EGFP. The full length from mTSLP/pEGFP-N1 was also subcloned into the *EcoR*I/*BamH*I sites of pHIgV vector (a kind gift from Dr. L. Chen, Johns Hopkins University School of Medicine, Baltimore, MD) for fusion protein mTSLP-hIg expression in HEK 293 cells. The reading frame of 121-amino acid (residues 20–141), which was deleted of the 19-amino acid signal peptide,[11] was amplified from the template plasmid mTSLP/pcDNA3.0 and cloned

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into the *EcoR*I/*BamH*I (TaKaRa) sites of pMAL-c2X vector (NEB, Ipswich, MA) for the fusion protein MBP-TSLP expression in bacteria. All the sequences were verified by DNA sequencing.

Cells and transfections

P815 cells were cultured with complete RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) containing 10% fetal calf serum (FBS; Gibco-BRL). HEK 293 cells were maintained in Dulbecco modified Eagle medium (DMEM; Gibco-BRL) containing 10% FBS, 25 mM HEPES, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate. The cells were transfected using the calcium phosphate method.

Recombinant protein expression

The construct of mTSLP/ pMAL-c2X was transformed into *E.coli* DH5α. Recombinant protein MBP-mTSLP was induced for 4 h at 37°C with 0.3 mM/L IPTG. The harvested cells were broken by sonication. MBP-mTSLP was purified with amylase resin (NEB), according to the manufacturer's instruction manual.

HEK 293 cells were transfected with mTSLP/pHIgV by the calcium phosphate method. The cells were re-fed with DMEM medium containing 0.1% FBS 12 h after transfection. The supernatant was collected and condensed with the Labscale TFF system (Millipore, Billerica, MA). Fusion protein mTSLP-hIg was purified with protein G affinity chromatography (Amersham Biosciences, Piscataway, NJ).

Animals and immunization

Female Wistar rats (Vital River Laboratory, Beijing, China) weighing 180 ~ 220 g were used for immunization. The immunization protocol of DNA priming-protein boosting was undertaken in this study. In brief, 0.75% bupivacaine was injected into the right and left tibialis cranialis muscles (i.m.) of the rats on day 0. Subsequently, the rats were immunized with 300 μg of plasmid mTSLP/pcDNA3.0 in 50 μL PBS on days 7 and 21, respectively. Fusion protein MBP-mTSLP (200 μg) was administered subcutaneously (s.c.) in proximity to draining lymph nodes as the boosting immunization on days 56 and 84, respectively. Initial boosting was performed using Freund's complete adjuvant (Sigma, Saint Louis, MO), while the second boosting was undertaken with incomplete adjuvant (Sigma). Pre-immune sera were obtained from the tail vein just before the first immunization to use as the negative control in specific antibody evaluation. Post-immune sera were also collected 10 days after the second fusion protein boosting and stored at −20°C. Enzyme-linked immunosorbent assay (ELISA) was performed to determine anti-TSLP titer in the sera. When the serum titer of TSLP antibody reached 10^{-5}, the rats were boosted last with 200 μg fusion protein MBP-mTSLP by intraperitoneal (i.p.) injections.

Hybridoma generation and antibody purification

Five days after the last intraperitoneal boosting, splenocytes from the immunized rat were fused with the myeloma cell line SP2/0 using PEG2000 (Sigma) as described.[12] The supernatants of the hybrid cell colonies with a surface growth greater than 20% were screened for antibodies by the ELISA method after 2 weeks. Positive cell colonies were subcloned by limiting dilution method (1 cell/well) three times.

Selected clones were expanded as ascites by intraperitoneal injection of 4×10^6 cells in 6 ~ 8 week-old female Balb/c nude mice (Vital River Laboratory) primed with pristine (Sigma). After 7–10 days, ascites were drained daily and the antibodies were purified using protein G (Amersham Biosciences) affinity chromatography. The isotype of the antibodies was determined using a rat monoclonal antibody isotyping kit (Roche, Basel, Switzerland).

ELISA assay

Antibody titer determination and antibody screening were conducted with the ELISA method. Briefly, the ELISA plate was coated with 5 μg/mL fusion protein mTSLP-hIg at 4°C overnight and blocked with blocking buffer (PBS, 10% fetal calf serum, 0.05% Tween-20 [PBS-T]) for 1 h. After washing with PBS-T, 100 μL antibody-containing supernatant was added and incubated for 2 h at room temperature. After another extensive washing, the secondary antibody of peroxidase-labeled goat anti-rat IgG (Sigma) diluted 1:10,000 in blocking buffer was added and incubated for 1 h before color development with the substrate of TMB (Pierce, Rockford, IL). The reaction was stopped by 2 N H_2SO_4 at the desired intensity and absorption at 450 nm was measured immediately with a microplated reader (Biolab, Ontario, Canada).

For epitope mapping, the purified antibody 4B12 was biotinylated with EZlinkSulfo-NHS-LC-Biotin (Pierce) before performing competitive ELISA. The wells coated with mTSLP-hIg were pre-incubated with an increasing amount of non-labeled 4E11, 5D7, and 6H10 (ranging from 0.03 to 300 μg/mL) for 45 min at 37°C. Non-biotin-labeled-4B12 was used as the competitive control. After washing, 10^{-4} dilutions (50 ng/mL) of the biotinylated 4B12 was incubated for 1 h at 37°C. After extensive washing, avindin-peroxidase (eBioscience, San Diego, CA) diluted 1:1000 in blocking buffer was added and incubated for 30 min at room temperature. The assay was reproduced in triplicates.

Flow cytometry analysis

HEK 293 cells were transiently transfected with a construct of mTSLP/pEGFP-N1. Cells at 1×10^6 were incubated with obtained antibodies in FACS buffer (3% fetal calf serum in PBS) for 30 min at 4°C. The cells were washed and further incubated with phycoerythrin (PE)-conjugated goat anti-rat IgG (eBioscience) for 30 min at 4°C. Fluorescence was analyzed with a FACSCalibur flow cytometry (Becton Dickinson, Mountain View, CA) with Cell Quest software (Becton Dickinson).

Intracellular staining assay

The cells were pretreated with Brefeldin A (eBioscience) 4 h before cell harvesting. Cells were harvested, fixed in 4% formaldehyde, and permeabilized in the permeabilization buffer (eBioscience). The following procedure was the same as the sample preparation of flow cytometry analysis.

Western blot analysis

Fusion protein of mTSLP-hIg was boiled with loading buffer and run on SDS-PAGE gel. After electrophoresis, the gel was transferred to polyvinylidene flvoride (PVDF) microporous membrane. The membrane was probed with anti-TSLP

antibodies at the concentration of 5 μg/mL for 1 h at room temperature. Protein hIg was used as the negative control. After extensive washing with TBS-T (20 mM Tris–HCl [pH 7.5]; 500 mM NaCl; 0.05% Tween-20), the membrane was incubated for another hour with peroxidase-conjugated goat anti-rat IgG (Sigma) diluted 1:8000 at room temperature. Chemiluminescent substrate (Pierce) was used to visualize the positive protein antigens. To determine the TSLP expression in the tissue, the mouse thymus was removed and homogenized in the low stringency buffer (10 mM Hepes [pH 7.6], 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM NaF, 5 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The supernatant was mixed with loading buffer. The following procedure was the same as above.

Results

Bacterial expression and purification of recombinant MBP-TSLP

The construct mTSLP/pMAL-c2X was transformed into the *E. coli* DH5α. After induction at 37°C for 4 h, a band with a molecular weight of about 57 kDa was detected by SDS-PAGE (Fig. 1A). The size approximated to the predicted molecular weight of fusion protein MBP-TSLP, which was purified with amylase resin, and the purity was analyzed by SDS-PAGE (Fig. 1B).

Sera titer analysis of antibodies

Wistar rats were immunized with DNA priming-protein boosting strategy. As shown in Figure 2A, the titer of antibodies of the rat post-immune sera were analyzed by the ELISA method. To ensure the facticity, fusion protein mTSLP-hIg other than MBP-TSLP (used for immunogen in this study) was used as the coated protein. The results showed that fusion protein mTSLP-hIg immobilized onto the ELISA plate can be detected by the serially diluted post-immune sera at the dilution ratio 10^5 or below. However, pre-immune sera were not effective in this detection system. This result indicated that titer induced by DNA priming-protein boosting mounted to 10^5 post-immunization.

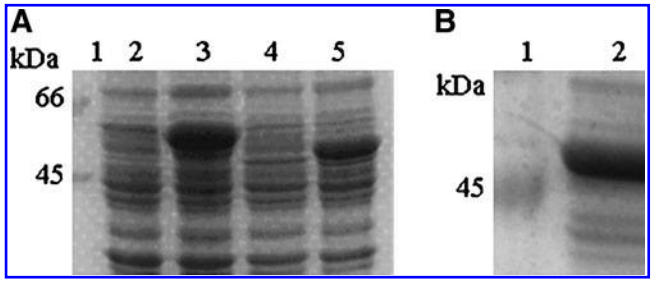

FIG. 1. Bacterial expression and purification of recombinant MBP-TSLP. **(A)** Fusion protein MBP-TSLP expression with pMAL-c2X system. DH5α lysates without IPTG induction (lane 2) and lysates with 4 h induction (lane 3) were separated by 12% gel and stained for 2 h with Coomassie Brilliant Blue G-250. Bacteria proteins transformed with pMAL-c2X were used as the control (lanes 4, 5). Lane 1 represented the mark. **(B)** SDS-PAGE analysis of the purified recombinant protein. Recombinant protein MBP-mTSLP was purified with amylase resin by affinity chromatography. The purity was assessed by SDS-PAGE analysis (lane 2).

Production and screening of MAbs

The rat with highest anti-TSLP titer was sacrificed and cell fusion was performed. 96-well plate fusions were cultured for 2 weeks. Supernatants of the hybridomas were screened by ELISA method. After three rounds of screening with mTSLP-hIg as antigen, four anti-TSLP MAbs with stable and high affinity were selected. These MAbs were referred to as 4B12, 4E11, 5D5, and 6H10. All of the MAbs were purified from mouse ascites and analyzed by SDS-PAGE. Two major bands with a molecular weight of ~50 kDa and ~30 kDa were observed, which corresponded to heavy chain and light chain of antibody, respectively (Fig. 2B). Their isotypes were all IgG2a, as determined by a rat antibody isotyping kit.

Specificity of MAbs

To further confirm the specificity of the obtained antibodies, the full length of TSLP was cloned into the modified pEGFP-N1 vector, in which a ~20 aa transmembrane fragment was inserted in the upstream of EGFP. Thus, TSLP can be anchored on the membrane of HEK 293 cells transfected with construct mTSLP/pEGFP-N1. After 36 h of transfection, cells were harvested and separated into two groups—fluorescence-positive cells (Fig. 3A, R2) and fluorescence-negative cells (Fig. 3A, R3). The latter negative cells were supposedly the non-transfected cells; therefore, these cells could be considered the negative control for the former

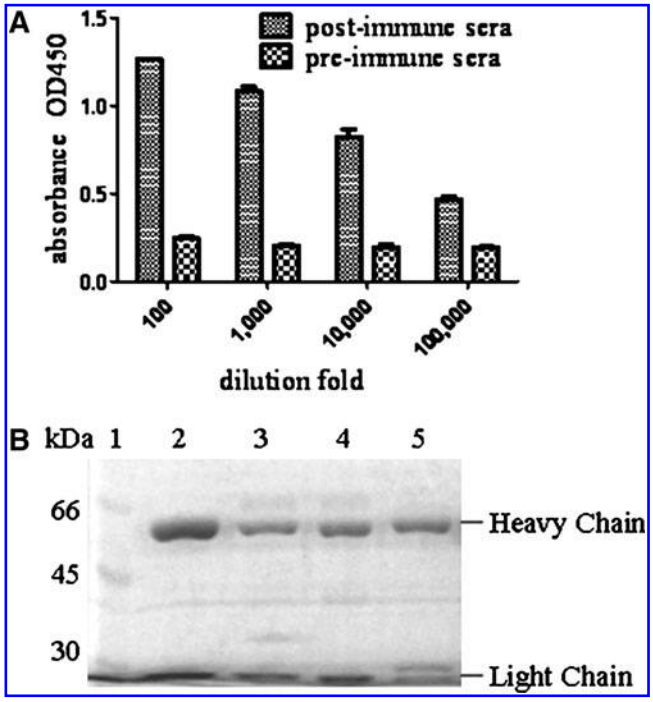

FIG. 2. Sera anti-TSLP titer analysis and determination of the purified TSLP antibodies. **(A)** Enzyme-linked immunosorbent assay (ELISA) was performed to determine anti-TSLP titer 10 days after second fusion protein boosting. Pre-immune sera were used as the negative control. **(B)** Selected clones were expanded as ascites by i.p. injection in BALB/c nude mice. After 7–10 days, ascites were drained daily and the antibodies were purified using affinity chromatography.

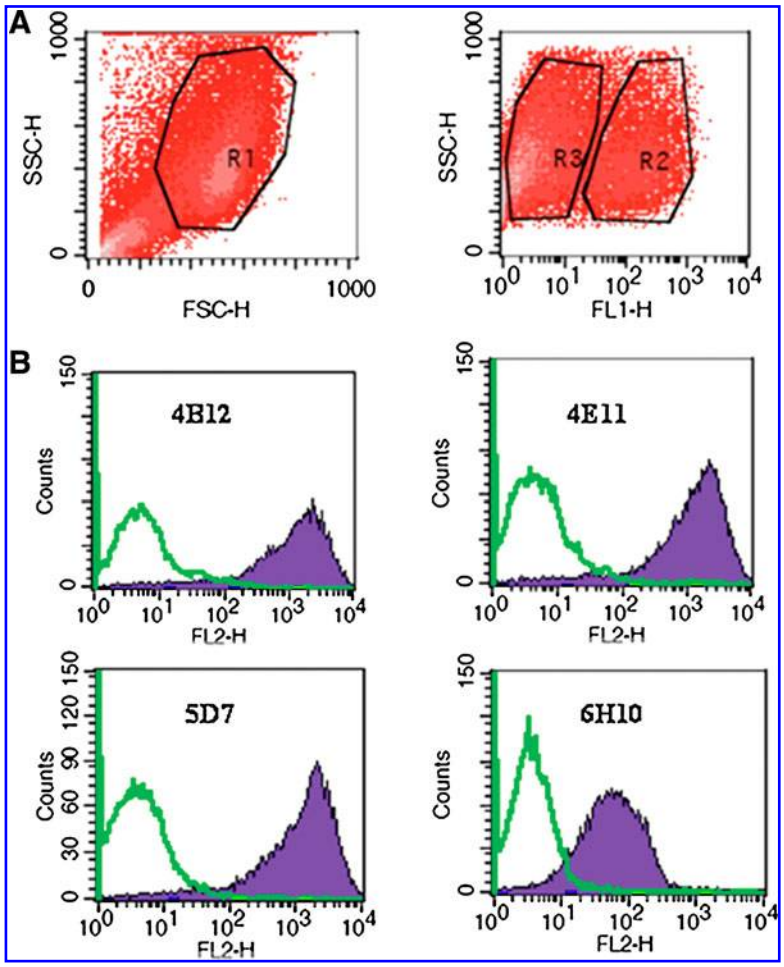

FIG. 3. FACS analysis of the specificity of TSLP antibodies. **(A)** HEK-293 cells (R1) transfected with construct mTSLP/pEGFP-N1 were separated into transfected (R2) and non-transfected cells (R3) according to intensities of GFP fluorescence 36 h after transfection. **(B)** The cells were stained with a panel of monoclonal antibodies and subjected to FACS analysis. The plots were gated on GFP-positive cells and GFP-negative cells, respectively. Open histogram, GFP negative cells; filled histograms, GFP positive cells. Results shown are from one representative of two independent experiments.

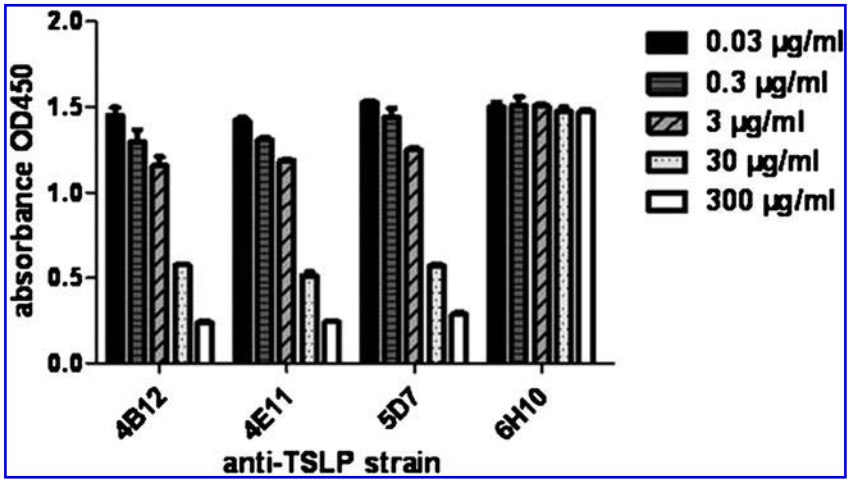

FIG. 4. Epitope mapping of the TSLP antibodies. Competitive ELISA was performed to determine the epitope of TSLP antibodies. 5 μg/mL fusion protein mTSLP-hIg was coated overnight at 4°C. An increasing amount of 4E11, 5D7, and 6H10 (ranging from 0.03 μg/mL to 300 μg/mL) were used as competitors of biotin-labeled 4B12 to bind the coated mTSLP-hIg.

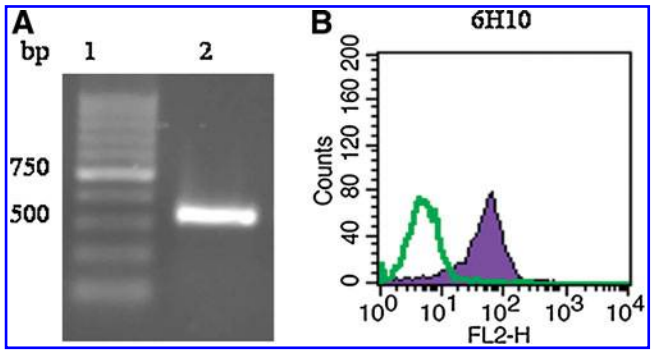

FIG. 5. Intracellular staining analysis with TSLP antibodies. **(A)** TSLP mRNA expression is observed in P815 cells by RT-PCR method. **(B)** Intracellular staining analysis was performed with TSLP antibodies in P815 cells. P815 cells were pretreated with Brefeldin A. 4B12 and 6H10 were used as the primary antibody, respectively. Rat IgG2a was used as the isotype control. PE-conjugated anti-rat IgG was employed as the secondary antibody. Samples were analyzed with a FACSCalibur flow cytometer using CellQuest software. Open histogram, isotype control; filled histograms, 6H10.

positive cells capable of expressing exogenous TSLP. As shown in Figure 3B, all four MAbs can bind to the fluorescence-negative cells, but not to the negative control cells, further providing the specificity of MAbs.

Epitope mapping

The competition ELISA method was developed to epitope mapping. Non-biotin-labeled 4E11, 5D7, and 6H10 were used as the competitors of the biotin-labeled 4B12, respectively. As is shown in Figure 4, both 4E11 and 5D7 competed with 4B12 in a dose-dependent manner, suggesting that these four MAbs were mapped onto two different epitopes of TSLP.

Intracellular staining assay of TSLP

Mast cells have been reported to express a great amount of TSLP.[13] P815, a mouse lymphoblast-like mastocytoma cell line, was used to determine whether these MAbs can be applied in the intracellular staining assay. First, we examined whether TSLP expressed as expected. RT-PCR analysis showed that TSLP expressed at the transcriptional level. However, immunofluorescence was only observed in the cells stained by 6H10 (Fig. 5B). The other three MAbs failed to recognize intracellular TSLP of fixed P815 cells (data not shown). It indicated that the epitope recognized by 4B2, 4E11, and 5D7 may be masked or denatured during routine formaldehyde fixation.

Western blot analysis of TSLP-specific MAbs

To test which strains can be used in Western blot analysis, fusion protein mTSLP-hIg purified from the supernatant of HEK 293 cells transfected with plasmid mTSLP/pHIgV was subjected to Western blotting. As shown in Figure 6A (lanes 2–5), the bands with a molecular weight of 42 kDa were detected with all four MAbs. The size of the bands approximated to the predicted molecular weight of this recombinant protein, whereas protein hIg (Fig. 6A, lane 1) cannot be detected by any strain of antibodies, suggesting that the four strains of anti-TSLP antibodies can all specifically recognize fusion protein mTSLP-hIg. Subsequently, mouse thymus lysates were used to determine whether these antibodies can be used to detect TSLP expression in the tissue. As shown in Figure 6B, 17 kDa bands were observed in lanes 1–4, which was consistent with the predicted molecular weight of TSLP. These results further indicated that 4B12, 4E11, 5D7, and 6H10 can be used in Western blot analysis.

Discussion

The aim of the present work was to produce specific antibodies against mouse thymic stromal lymphopoietin. TSLP is an interleukin IL-7-like cytokine that triggers dendritic cell-mediated T helper (Th)2 inflammatory responses.[1] TSLP is highly expressed by keratinocytes in skin lesions of patients with atopic dermatitis and is associated with dendritic cell activation *in situ*, suggesting that TSLP might be a master switch for allergic inflammation at the epithelial cell-dendritic cell interface.[13] New reports now establish a direct link between TSLP expression and the pathogenesis of atopic dermatitis and asthma *in vivo*.[10] We now know that TSLP is highly expressed by skin keratinocytes and airway epithelial cells during allergic inflammation. However, how TSLP expression is triggered in these cells—by allergen exposure or by virus infection—remains unclear. Most of the commercial antibodies available against TSLP can only be used in Western blot assay, which significantly prevents further investigation

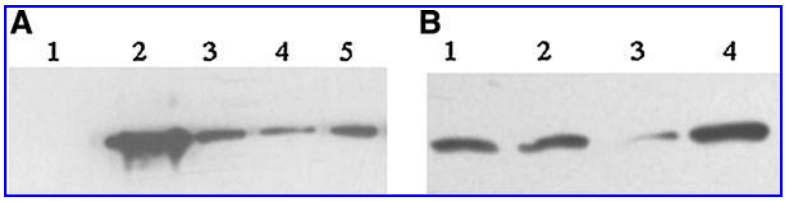

FIG. 6. Western blot analysis of TSLP antibodies. **(A)** Fusion protein mTSLP-hIg purified from the supernatant of HEK 293 cells transfected with plasmid mTSLP/pHIgV was subjected to Western blotting. 4B12 (lane 2), 4E11 (lane 3), 5D7 (lane 4), and 6H10 (lane 5) were used as the primary antibodies, respectively. Protein hIg was used as the negative control. Peroxidase-conjugated goat anti-rat IgG2a diluted 1:8000 was used as the secondary antibody. Chemiluminescent substrate was used to visualize the positive protein antigens. **(B)** Lysates prepared from mouse thymus tissue were subjected to Western blotting using 4B12 (lane 1), 4E11 (lane 2), 5D7 (lane 3), and 6H10 (lane 4), respectively.

of TSLP. In this study, we successfully generated a panel of anti-TSLP antibodies through immunizing rats with DNA priming-protein boosting strategy.

Four strains of MAbs against mouse TSLP (4B12, 4E11, 5D7, and 6H10) all can significantly bind to mouse TSLP. FACS analysis using HEK 293 cells transfected with mTSLP/pEGFP-N1 further confirmed the specificity of the four anti-TSLP antibodies. According to competitive ELISA results, these four MAbs map two different epitopes. Only 6H10 can recognize the epitope located on the fixed P815 cells in the intracellular staining assay while the other three MAbs failed to recognize intracellular TSLP of fixed P815 cells. It is indicated that the epitope recognized by 4B12, 4E11, and 5D7 may be masked or denatured during routine formaldehyde fixation. Western blot analysis demonstrates that these four MAbs not only can recognize the purified recombinant mTSLP-hIg, but also can detect TSLP expression in the mouse thymus lysate.

To sum up, this study generated and characterized a panel of monoclonal antibodies against two distinct epitopes of mouse TSLP. All four antibodies can be used in Western blot analysis, and especially 6H10 can be used in the intracellular staining assay. To our knowledge, it is the first time an anti-TSLP antibody has been obtained that can be employed in the intracellular staining assay. Thus, these antibodies provide valuable tools for studying TSLP expression and function.

Acknowledgments

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