Development and characterization of monoclonal antibody against human IL-37b

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Abstract IL-37 has been described as a natural inhibitor of immune responses. Monoclonal antibody (mAb) against human IL-37b with high affinity and specificity can serve as a molecular probe to detect IL-37 and study IL-37 functions, mechanisms and related signal pathways in inflammatory diseases. However, there are very few such mAbs against human IL-37 commercially available so far. In the current study, monoclonal antibodies against human IL-37b were developed by fusing splenocytes from immunized

mouse with SP2/0 myeloma cells and polyethylene glycol. Then the antibodies were screened with prokaryotic expressed human IL-37b protein and eukaryotic expressed human IL-37b protein subsequently. Western blot and flow cytometry analysis revealed that selected mAb clons were able to recognize human IL-37 with high specificity. And more importantly, the IL-37b mAbs were fluorescently labeled and can be directly used in flow cytometry and immunohistochemistry. In conclusion, the current study developed new mAbs against human IL-37b, which are applicable in flow cytometry and immunohistochemistry.

Yu-Chi Gao, Yan Jia, De-Qian Xiao have contributed equally to this work.

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Introduction

Interleukin-37 (IL-37), a newly discovered member of the IL-1 family, has been identified as a natural inhibitor of immune responses (Nold et al. 2010). IL-37 has five splice variants including IL-37a–e (Boraschi et al. 2011), with only IL-37b and IL-37c expressing an N-terminal caspase-1 cleavage site (Wu et al. 2016). IL-37b is the longest and most studied isoform, with a significant sequence similarity to IL-18. Increasing evidence indicates that the modulation of immune system by IL-37 involves the suppression

of both innate and adaptive immunity (Boraschi et al. 2011; Chen and Fujita 2015; Ye et al. 2014). It has been shown that IL-37 down-regulates the expressions of pro-inflammatory cytokines in chronic inflammatory diseases (Ye et al. 2015), suggesting that IL-37 might abrogate pro-inflammatory cytokines productions and reduce inflammatory responses. Regulation of IL-37 expression may provide a new possible target for diagnosis and treatment of inflammatory diseases (Ballak et al. 2014; Fonseca-Camarillo et al. 2015).

Antibodies are key tools in basic study and in clinical diagnosis and treatment. Although there are some monoclonal antibodies (mAbs) against human IL-37 commercially available, it is necessary to prepare more mAbs for human IL-37 for use in studies and clinical applications. In this study, we used prokaryotic expressed soluble human IL-37b recombinant protein to immunize BALB/c mouse. Then hybridoma cell lines secreting specific monoclonal antibodies against human IL-37 were generated successfully. The specificity of mAb from one hybridoma cell line 1C6 was confirmed by Western blot, flow cytometry, and immunohistochemistry.

Materials and methods

Reagents and materials

Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Freund's adjuvant (complete and incomplete), HT (hypoxanthine, thymidine), HAT (hypoxanthine, aminopterin, thymidine), goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody, and polyethylene glycol 4000 (PEG4000) were from Sigma Chemicals (St. Louis, MO, USA). Commercial anti-IL37 antibody was purchased from Abcam (Abcam, Cambridge, MA, USA). Mouse IgG1 Isotype Control, Alexa Fluor® 647 was purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, USA). Allophycocyanin (APC)-conjugated goat anti-mouse secondary antibody was from BD (Franklin Lakes, NJ, USA). Protein G Sepharose column was purchased from GE Healthcare (Uppsala, Sweden). Phosphate buffer saline (PBS), TROZOL (TaKaRa, Otsu, Shiga, Japan), isopropanol, chloroform, 75% ethanol, TBE buffer, agarose, total protein extraction kit (BestBio, ShangHai, China), PrimeScript™ RT Master Mix

(TaKaRa), TaKaRa EX Taq (TaKaRa), *E. coli* DH5 α (TaKaRa), Gel Recovery Kit (TaKaRa), TA cloning kit (Qiagen, Hilden, Germany) were purchased commercially.

Soluble human IL-37b protein prokaryotic expression system (pET28a/IL-37 in *E. coli* BL21) (Zhao et al. 2014a) and human IL-37b-GFP fusion protein eukaryotic expression system (pcDNA3.1-il37-GFP) (He et al. 2015) were constructed in our laboratory. Sp2/0-Ag14 cell line was from ATCC (CRL-8287™) (Manassas, VA, USA). Human embryonic kidney 293-T cell line was obtained from the Cell bank of Chinese Academy of Sciences. BALB/c mice (6 weeks old, female) were obtained from the Animal Experiment Center of Southern Medical University of China (DongGuan, China). All animals were strictly handled according to the Good Animal Practice Requirements of the Animal Ethics Procedures and Guidelines of the People's Republic of China. The present study was approved by the Animal Ethics Committee of the Institute of Laboratory Medicine, Guangdong Medical University (Approval no. 2014005). Human tissues were acquired from the First Affiliated Hospital of GDMU under warranty that appropriate IRB approval and informed consent was obtained from all human subjects before the study.

Preparation of recombinant human IL-37b proteins

Recombinant soluble human IL-37b expression was induced in *E. coli* BL21 (pET28a/IL-37) (Zhao et al. 2014a), with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 30 °C, and purified with a Ni²⁺ + Sepharose column (GE Healthcare), then confirmed through a 12% SDS-PAGE gel stained with Coomassie brilliant blue. Recombinant human IL-37b-GFP fusion protein was prepared by transfecting 293-T cells with the pcDNA3.1-il37-GFP plasmid. The expression of IL-37b-GFP fusion protein was confirmed under the fluorescence microscope. Then, the cells were lysed in 1 ml RIPA solution (containing 1% Triton X-100, 1% deoxycholate, and 0.1% SDS; Beyotime Biotechnology, Haimen, China) supplemented with a protease inhibitor (PMSF, Sangon, Shanghai, China) for 30 min at 4 °C, then centrifuged at 12,000g for 30 min at 4 °C to obtain the total cell extracts. The protein concentration in the cell extracts was determined by using a BCA protein assay kit (Beyotime).

Preparation, screening and purification of mAbs

The immunization, mAb production and screening were performed as described elsewhere (Yang et al. 2002). Briefly, healthy BALB/c mice were subcutaneously injected with recombinant human IL-37b protein fully emulsified with Freund's complete adjuvant and were boosted 3 times with IL-37b in Freund's incomplete adjuvant. Antibody titers were tested by indirect ELISA. Then, splenocytes were isolated and fused with SP2/0 in 50% PEG and cultured in HAT medium. Afterwards, hybridomas were screened by indirect ELISA using prokaryotic expressed soluble human IL-37b protein, and specificity of mAbs was identified by Western blotting and flow cytometry against recombinant human IL-37b-GFP fusion protein. Highly specific and high titer mAbs were produced in mouse further. Hybridoma cell lines secreting IL-37b mAbs with the higher titer were intraperitoneal injected. Then the ascites were collected and one of the hybridoma cells 1C6 with highest antibody titer was selected for purification using a Protein G Sepharose column according to the manufacturer's protocol.

Identification of mAbs

The class and subclass of the mAbs were identified by a mouse monoclonal antibody isotyping reagent (Life Technologies) according to the manufacturer's directions. Purified mAb 1C6 was analyzed by SDS-PAGE, while the titer was detected by indirect ELISA.

Detections of its antibody high variable region gene and amino acid sequence were carried out by PCR and

gene sequencing according to previous reports (Couto et al. 1993; Ye et al. 2013). Briefly, Total cellular RNA was isolated from pelleted hybridoma cells 1C6 with Trizol reagent. cDNA was synthesized by using oligo (dT) primers and reverse transcriptase. Then, cDNA was amplified by polymerase chain reaction (PCR) with specific primers designed by Abace Biotech (Abace Biotech, BeiJing, China) of IgG1 to amplify heavy and light chains (HC and LC). PCR products were confirmed with 2% agarose gel and then linked with T carrier directly and cloned into *E. coli* DH5 α (TaKaRa) using a TA cloning kit (Qiagen) according to the instruction. Afterwards, DNA was sequenced and analyzed with IMGT BLAST.

Then the specificity of the purified antibodies was identified. Firstly, purified mAb 1C6 was identified by Western blot against recombinant human IL-37b-GFP fusion protein (Zheng et al. 2016). 293-T Cells transfected with the pcDNA3.1-il37-GFP plasmid were lysed and directly loaded for the SDS-PAGE analysis. Proteins were electrophoretically transferred to PVDF membranes (EMD Millipore, Merck KGaA, Darmstadt, Germany). The membranes were incubated with an optimal concentration of IL-37b mAb overnight at 4 °C, and further incubated with HRP-conjugated goat anti-mouse IgG antibody. Proteins of interest were visualized with ECL in the FluorChemTM FC3 system (Protein Simple, Rochester, NY, USA).

Secondly, purified mAb 1C6 was verified by flow cytometric detection of its binding with recombinant human IL-37b-GFP fusion protein. As IL-37b is expressed intracellularly, the binding of IL-37b-GFP fusion protein with antibodies was detected by intracellular cytokine staining method (Zeng et al. 2015).

Fig. 1 Analysis and identification of human IL-37. Prokaryotic expressed soluble human IL-37 protein served as immunogen to raise mouse anti-human IL-37 monoclonal antibody, and eukaryotic expressed human IL-37-GFP fusion protein was used as identification antigen to screen the mAbs. **a** Shows purity of soluble human IL-37 protein analyzed by SDS-PAGE. *M* Protein marker; *1* no IPTG induction; *2* 1 mM IPTG

induction; *3* precipitation of an extract of expressing bacteria after ultrasonic fragmentation; *4* supernatant of the expressing bacteria after ultrasonic fragmentation; *5* purified human IL-37. **b** Shows human IL-37 expression in 293-T cells transfected with pcDNA3.1-il37-GFP. **c** Western blotting analysis of human IL-37 expressed in 293-T cells using a commercial anti-human IL-37b mAb

Fig. 2 Western blotting of prokaryotic expressed soluble human IL-37 protein and eukaryotic expressed human IL-37-GFP fusion protein with antibodies against human IL-37. Soluble IL-37 expressed in *E. coli* and total proteins of 293-T cells transfected with pCDNA3.1-il37-GFP were determined by western blot using antibodies against human IL-37, and the

mock-vehicle without IL-37 genetic fragments transfected *E. coli* and 293-T cells were used as a negative control in all experiments. The sizes of the marker proteins are listed on the left. **a** Selected antibody 1C6 and 1G7 reacted with soluble IL-37 expressed in *E. coli*. **b** Antibodies reacted with total proteins of 293-T cells transfected with pCDNA3.1-il37-GFP

Table 1 Characterization of selected clones in terms of titer, Ig class, and Ig subclass

Hybridoma	Class and subclass	Titer of supernatant of cell culture medium
1C6	IgG1, K	51,200
1D4	IgG2a, K	25,600
1D10	IgG1, K	12,800
2A3	IgG2a, K	25,600
2D1	IgG1, K	25,600
2E9	IgG1, K	25,600
2F5	IgG1, K	12,800
2H12	IgG1, K	51,200

MAbs were fluorescently labeled using an Alexa Fluor® 647 Monoclonal Antibody Labeling Kit according to manufacturer's instructions. Then, 293-T cells transfected with pCDNA3.1-il37-GFP were stained with labeled IL-37b mAb intracellularly. Briefly, cells were collected and transferred into 5 ml polystyrene round bottom tubes (BD Pharmingen, San Jose, CA, USA) for staining. Then, the cells were washed once with 2% FBS-PBS and permeabilized with BD FACS permeabilizing solution (BD Pharmingen) for 30 min at room temperature, and then stained for 45 min for IL-37b mAb followed by two final 2% FBS-PBS buffer washes and analyzed on a BD FACS Canto™ II flow cytometer. To ensure the specific immune staining in ICS, matched isotype IgG served as negative controls for staining cytokines. Data were

Fig. 3 Flow cytometric analysis of antibodies against human IL-37 reacting with (eukaryotic expressed) human IL-37-GFP fusion protein. Specificity of antibodies against human IL-37 was analyzed by indirect fluorescent staining with flow cytometry using pCDNA3.1-il37-GFP transfected 293-T cells. Firstly, pCDNA3.1-il37-GFP transfected 293-T cells reacted with either in the absence (red lines) or presence (blue lines) of antibodies, then stained with APC labeled second antibody against mouse IgG, intracellularly. **a** pCDNA3.1 transfected 293-T cells. **b** pCDNA3.1-il37-GFP transfected 293-T cells. **c–j** Antibodies against IL-37 reacted with IL-37 expressed in 293-T cells

analyzed with FlowJo flow cytometry analysis software.

Lastly, purified mAb 1C6 was identified by immunohistochemistry. As we found human cervical cancer tissue expressing IL-37b (data not shown), they were selected to detect IL-37b expression in natural state as previously reported (Zeng et al. 2013). Cervical cancer tissues were provided by Guangdong Provincial Hospital of Traditional Chinese Medicine (Guangzhou 510120, China). After fixation, tissues were washed in tap water, dehydrated in a graded alcohol series, embedded in paraffin, and cut into 3 mm thick sections. The paraffin sections were processed for hematoxylin-eosin staining and immunohistochemistry using the IL-37b mAb and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). IL-37b immuno reactive areas were observed using an inverted microscope.

Results

Preparation of recombinant human IL-37b proteins

In this study, prokaryotic expressed soluble human IL-37b protein was used as immunogen to raise mouse anti-human IL-37b antibody, and human IL-37b protein expressed in 293-T cells served as screening antigen. Soluble human IL-37 protein was expressed with pET28a/IL-37 in *E. coli* BL21 system (He et al. 2015). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis confirmed that the product is a 25 kDa soluble protein, which is consistent with the predicted molecular weight of the recombinant protein, with high purity (Fig. 1a). Meanwhile, fluorescence microscopy showed that IL-37-GFP fusion protein was highly expressed in 293-T cells (Fig. 1b) and Western blotting using a commercial human IL-37b mAb showed a band at 42 kDa which corresponds to the IL-37-GFP fusion protein (Fig. 1c).

Screening of hybridomas secreting anti-human IL-37b mAbs

Hybridomas secreting anti-human IL-37b mAbs were screened with (prokaryotic expressed) soluble recombinant human IL-37b protein by indirect ELISA (Fig. 2a). A total of 30 colonies showed positive reaction with human IL-37b (data not shown). Then, Western blot was employed to screen the mAbs with eukaryotic expressed human IL-37 protein extracted from pCDNA3.1-il37-GFP transfected 293-T cells. The results showed that 17 colonies could secrete mAbs specifically reacting with human IL-37b (Fig. 2b).

However only 8 colonies (designated as: 2A3, 1C6, 2D1, 1D4, 1D10, 2E9, 2F5 and 2H12) producing high titers were successfully subcloned and cultured. Interestingly, only four hybridoma clones (2E9, 2F5, 1C6 and 2D1) could secrete antibody specifically reacting with human IL-37b both in flow cytometry and in Western blot analysis (Fig. 3c–f).

Ig class and subclass of mAbs

Table 1 summarizes the class, and subclass of the selected clones and antibody titers in the cell culture supernatant of the clones, demonstrating that the antibodies all belong to IgG1 (1C6, 2D1, 1D10, 2E9, 2H12 and 2F5) or IgG2 (1D4 and 2A3), subclass with kappa light chain (Table 1).

Purity and titer of purified IL-37b mAb 1C6

As 1C6 has the highest titer among all of the clones, it was selected to produce mAb. Protein G Sepharose column purification resulted in a concentration of 250 µg/ml for the mAb. SDS-PAGE analysis shows two bands in lanes 1, 2 and 3 with molecular weight of 50 kDa and 25 kDa, which correspond to the molecular weights of Ig heavy chain and light chain (Fig. 4a). Indirect ELISA analysis showed that the titer in ascites was 2.04×10^7 (Fig. 4b), and purified antibody titer was 1.28×10^6 (Fig. 4c).

High variable region sequence of 1C6 antibody

HC and LC genes of 1C6 were amplified by RT-PCR using different degenerate primers. The intact HC

Fig. 4 Purity and titer analysis of the purified IL-37 mAb 1C6. Hybridoma cell 1C6 was inoculated into the peritoneum of BALB/c mouse, then anti-human IL-37 mAb was purified from ascites with protein G affinity chromatography and its purity was analyzed by SDS-PAGE, while titer of the mAb as tested by indirect ELISA. **a** SDS-PAGE determinant of 1C6 antibody.

M Protein marker; *lane 1* 10 µl purified IL-37 MAB; *lane 2* 13 µl purified IL-37 MAB; *lane 3* 16 µl purified IL-37 MAB. **b** Titer detection of before purification 1C6 antibody in ascites: 2.04×10^7 ; **c** Titer detection of purified 1C6 antibody: 1.28×10^6

Fig. 5 Analysis of the variable region sequence of 1C6 antibody. Variable region sequence of 1C6 antibody was determined by RT-PCR by Creative BioLabs (45-16 Ramsey Road Shirley, NY 11967, USA). **a** Agarose gel electrophoresis of RT-PCR amplification of intact HC gene. Lane *M* DL2000 DNA marker; lane *H* PCR production of HC using heavy chain primer. **b** Agarose gel electrophoresis of RT-PCR amplification of intact LC gene. Lane *M* DL2000 DNA marker; lane *L* PCR product using light chain primer. **c**, **d** Show variable region gene and protein sequence of heavy and light chains, respectively. The framed regions indicate CDRs

Fig. 6 Identification of the specificity of the purified mAb 1C6 by Western blot and flow cytometry against recombinant human IL-37b-GFP fusion protein. Western blot and flow cytometry were employed to validate the specificity of mAb 1C6 in detection of human IL-37. **a** Western blot detection. *M* Protein marker; *Lanes 1–4* antibody at concentrations of 0.5, 1, 2 and 5 $\mu\text{g/ml}$, respectively. **b** Flow cytometric analysis of 293-T cells transfected with pcDNA3.1 or pcDNA3.1-il37-GFP. **c–g** IL-37 expression in 293-T cells analyzed by flow cytometry with different concentrations of 647-1C6 mAb. The concentrations were as following: 0.125, 0.25, 0.5, 0.75 and 1 $\mu\text{g}/10^6$ cells. *I* Mouse IgG1 Isotype Control and 2 mAb 1C6

gene is 1306 bp and LC gene is 631 bp with correct primers (Fig. 5a, b). Variable gene and protein sequence of heavy and light chain were analyzed. CDRs sequences in VH and VL are shown in Fig. 5c, d.

Identification of the specificity of the purified 1C6 mAb

Western blot analysis showed that purified IL-37 mAb 1C6 could react with the total protein extracted from pcDNA3.1-il37-GFP transfected 293-T cells with

molecular weight of about 42 kDa corresponding to the molecular weight of IL-37-GFP fusion protein (Fig. 6a). Meanwhile, flow cytometry analyzed by direct intracellular cytokine staining (ICS) showed purified IL-37 MAb 1C6 could also react with pcDNA3.1-il37-GFP transfected 293-T cells in a concentration dependent manner. Notably, immunohistochemical staining showed that purified IL-37 MAb 1C6 could react with endogenous human IL-37 expressed in cervical cancer tissue with the same pattern as a commercial MAb against human IL-37 (Fig. 7).

Discussion

The present study successfully generated 8 hybridomas which secrete mAbs against human IL-37b. All of the 8 mAbs could react with recombinant human IL-37b protein expressed in 293-T cells via Western blotting, and 4 mAbs could react with recombinant human IL-37b protein expressed by 293-T cells both

Fig. 7 Identification of the specificity of the purified mAb 1C6 by immunohistochemistry against endogenous IL-37 in cervical carcinoma tissue. IHC was employed to validate the specificity of MAb 1C6 in detection of endogenous human IL-37 in tumor

tissue. **a** Immunohistochemical staining of cervical carcinoma tissue with commercial antibody against human IL-37. **b** Immunohistochemical staining of cervical carcinoma tissue from the same patient as in **a** with the 1C6 MAb

in Western blotting and in flow cytometry. Hybridoma 1C6 produced the highest titer among the 8 clones and was used to produce mAb and further purified (data not shown). It was confirmed that 1C6 mAb specifically binds to human IL-37 in Western blot, flow cytometry and immunohistochemistry.

IL-37 is involved in the suppression of innate and adaptive immunity (Boraschi et al. 2011; Chen and Fujita 2015; Ye et al. 2014). Recent reports indicate that IL-37 plays important roles in inflammatory disease (Charrad et al. 2016; Ye et al. 2014; Zhao et al. 2014b), infectious disease (Anuradha et al. 2016; Hojen et al. 2015; Samarani et al. 2016), cancer (Ding et al. 2016; Ge et al. 2016) and angiocardopathy (Huang et al. 2016; Yu et al. 2016). However, the exact mechanisms of IL-37 in human disease remain to be further studied. Antibodies against human IL-37 are a crucial and important tool to study IL-37b expression level, localisations, and receptors, and will facilitate the discovery of the physiological and pathophysiological functions of this molecule. Likewise, antibodies against human IL-37b will also be used to determine soluble IL-37b in the peripheral blood and other body fluid for the auxiliary diagnosis of human diseases. The present study successfully developed mAbs against human IL-37b, which laid the crucial foundations of further research on IL-37b in human diseases.

The immunogen is the most important substance in mAb development. In the present study, recombinant prokaryotic expressed soluble human IL-37 was used to immunize BALB/c mouse, and served as preliminary screening antigen. This protein is cost effective for large-scale production in comparison with the expensive and time-consuming eukaryotic expression system. While prokaryotic expressed protein can display the native confirmation, it could be sufficient to ensure that the mAbs have relatively high affinity and, in addition, that they can be applied for flow cytometry, Western blot and immunohistochemistry. We observed a high immunologic response in an immunized mouse, whose serum titer resulted in 1/1,280,000 in indirect ELISA, and all of the immunized mice could be selected for the cell fusion. Furthermore, eukaryotic expressed human recombinant IL-37b-GFP was used for antibody screening via Western blotting and flow cytometry. The results demonstrated that mAbs from 17 hybridoma clones

could react with IL-37b-GFP in Western blot, but only 8 clones could react also in flow cytometry analysis.

In the present study, we produced and purified mAb 1C6 from ascitic fluid at large-scale with high titer ($1/1.28 \times 10^6$) and a concentration of 1.9 mg/ml was obtained. This mAb can react with eukaryotic expressed human recombinant protein IL-37b-GFP in Western blotting and in flow cytometry. Notably, 1C6 can specifically recognize endogenous human IL-37 expressed in cervical cancer tissue tested by immunohistochemistry. These data demonstrate the potential of 1C6 mAb to be used as a diagnostic reagent for studying IL-37 expression in tumors.

In conclusion, we developed eight human IL-37 eukaryotic protein that can be used for the flow cytometry, western blot, immunohistochemical staining and Enzyme-linked Immunoassay. Importantly, we prepare the fluorescently-tagged 1C6 mAb. It may supply a more convenient path to detect IL-37 in further studying for functions, mechanisms, and related signal pathways of IL-37 in inflammatory diseases.

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Compliance with ethical standards

Conflict of interest All authors have no competing financial interests.

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