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ScienceDirect
Veterinary Immunology and Immunopathology 121 (2008) 359–363
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Rapid communication

Monoclonal antibodies reactive with chicken interleukin-17

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Received 4 May 2007; received in revised form 28 July 2007; accepted 5 October 2007

Abstract

Chicken interleukin-17 (chIL-17) gene was previously characterized through cloning from a chicken intestinal expressed sequence tag (EST) cDNA library. To further investigate the biological properties of chIL-17, six monoclonal antibodies (mAbs) against a bacterially expressed chIL-17 recombinant protein were produced and their binding specificities characterized. Antibodies which were initially selected on the basis of their specific binding reactivity with recombinant chIL-17 in ELISA were further characterized by Western blot analysis. Monoclonal antibodies specific for chIL-17 identified 20 and 21 kDa protein bands in the culture supernatant and cell lysate of CU205 cells. These mAbs also recognized specific bands for chIL-17 in the cell lysate from conconavalin A (Con A)-activated, but not from normal splenic lymphocytes. Furthermore, these mAbs detected a 16 kDa protein in the lysate of CU205 cells treated with tunicamycin and stained an intracellular protein in CU205 cells in flow cytometric analysis. Together, these results indicate that these new mAbs are specific for chIL-17 and will be a useful tool for structural and immunological studies of IL-17 in poultry.

Keywords: Chickens; Interleukin-17; Monoclonal antibody

IL-17 (IL-17A) was originally cloned from an activated T-cell hybridoma and exhibited similarity to a sequence belonging to the *Herpesvirus saimiri* (HSV13) gene product (Rouvier et al., 1993). Recently, the IL-17 cytokine family (IL-17A-F) has subsequently been defined with very distinct expression patterns and biological functions (Kolls and Linden, 2004). Furthermore, IL-17-producing T cells have recently been classified as a new lineage of effector T-cell subset and designated as the Th17 lineage distinct from the Th1 and Th2 lineages (Stockinger and Veldhoen, 2007; Weaver et al., 2007).

IL-17 is primarily secreted by activated $CD4^+$ T cells and has been implicated in autoimmune and inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, gastritis, bronchial asthma, and allograft rejection (Andoh et al., 2007). In addition, IL-17 has been implicated in host defense against bacteria, parasite and virus including *Mycoplasma pneumoniae*, *Taxoplasma gondii*, HIV, and *Eimeria* (Min and Lillehoj, 2002; Kelly et al., 2005; Maek-A-Nantawat et al., 2007; Wu et al., 2007).

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0165-2427/$ – see front matter
doi:10.1016/j.vetimm.2007.10.004

In chickens, IL-17 gene was initially cloned from a chicken EST cDNA library prepared from intestinal intraepithelial lymphocytes (IELs) of *Eimeria*-infected chickens (Min and Lillehoj, 2002). The role of chIL-17 in local host defense against *Eimeria* has been investigated and the transcript expression levels in *Eimeria*-infected chickens were measured (Hong et al., 2006). However, due to low sequence homologies between avain and mammalian cytokines (Staeheli et al., 2001; Min and Lillehoj, 2002), detailed studies of immunological function of many chicken cytokines including IL-17 are hindered by lack of specific immunological reagents. Thus, it is necessary to generate antibodies which are specific for chIL-17 that can be used for the detection and functional studies of chIL-17. In this paper, we describe the development and characteristics of mouse mAbs specific for chIL-17.

To express chIL-17 gene, the coding region of chIL-17 gene was cloned by PCR from chIL-17 gene (Min and Lillehoj, 2002) using the following primers (BamHI and HindIII restriction sites are underlined):

- sense primer, 5′-GATCGGATCCGCAGATGCTGG-ATGCCTAACC-3′;
- antisense primer, 5′-GATCAAGCTTCCTTTAAGC-CTGGTGCTGGAT-3′.

The PCR product was digested with BamHI and HindIII and inserted into the corresponding sites of the pMAL-c2X expression vector (New England Biolabs, USA) to form a sequence encoding maltose binding protein (MBP)harboring chIL-17 protein. The recombinant plasmid was transformed into competent *E. coli* DH5α and transformants were selected on ampicillin plates. *E. coli* colonies harboring recombinant plasmid were grown at 37 °C to $OD_{600} = 0.5$, induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C and disrupted by sonication on ice (Misonix, Farmingdale, NY, USA). The recombinant protein (MBP-chIL-17) was purified on amylose affinity column (New England Biolabs, USA) according to the manufacturer's instructions.

To generate B cell hybridomas secreting mouse mAbs against chIL-17, BALB/c mice were immunized subcutaneously with 50 μg of bacterially expressed MBP-chIL-17 mixed in Freund's complete adjuvant (Sigma–Aldrich, USA) and boosted twice biweekly with 50 μg MBP-chIL-17 in incomplete Freund's adjuvant. Two weeks post the final boost, mice were injected intravenously with 10 μg of MBP-chIL-17 in PBS. Splenocytes were collected 3 days later and fused with SP2/0 myeloma cells. Hybridomas producing chIL-17 mAbs were identified by ELISA as described (Min et al., 2002). Briefly, flat-bottom 96-well microtiter plates (Costar, USA) were coated with 100 μl of bacterially expressed affinity purified MBP-chIL-17 (5 μg/ml) in 0.1 M carbonate buffer, pH 9.6 at 4 °C overnight and washed three times with PBS containing 0.05% Tween 20 (PBS-T). Non-specific binding was blocked with 200 μl of PBS containing 1% bovine serum albumin (PBS-BSA) for 1 h at room temperature, the plates were washed with PBS-T, and incubated with 100 μl of hybridoma supernatant for 1 h at room temperature. The bound antibody was detected at OD_{450} after reacting with horseradish peroxidase conjugated goat anti-mouse IgG antibody (Sigma–Aldrich) and tetramethylbenzidine (USB, USA). Positive hybridomas were cloned by limiting dilution using mouse thymus feeder cells and isotypes determined using a commercial kit, ISO-2 (Sigma–Aldrich).

For Western blotting, protein samples were mixed with equal volumes of sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.004% bromophenol blue), heated for 4 min at 94 °C, resolved on 8–12% SDS-polyacrylamide gels and electroblotted to Hybond-P membranes (Amersham Biosciences, USA). The membranes were blocked with PBS containing 1% nonfat dry milk for 16 h at 4 °C, incubated with undiluted hybridoma culture supernatants, washed with PBS-T and bound antibody allowed to react with peroxidase conjugated rabbit anti-mouse IgG antibody in PBS-BSA for 40 min at room temperature. The membrane was washed five times with PBS-T and five times with distilled water and developed using 3,3′-diaminobenzidine substrate (Sigma–Aldrich).

A reticuloendotheliosis virus (REV)-transformed chicken lymphoblast cell line, CU205, and chicken

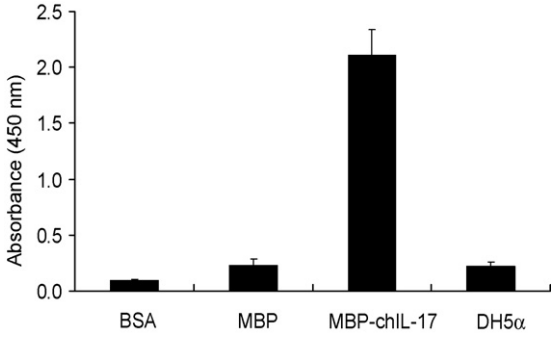

Fig. 1. The specificity of mAb 3D11 was analyzed by indirect ELISA. Flat-bottom 96-well microtiter plates were coated with 0.5 μg of BSA, MBP, MBP-chIL-17 and DH5α. *E. coli* DH5α was disrupted by sonication. Each point represents the means ± S.D. of triplicate determinations.

splenic lymphocytes which were isolated by Ficoll-Paque™ (Amersham Biosciences, Sweden) were cultured in Dulbecco's modified eagle's medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate (all from Sigma–Aldrich). Splenic lymphocytes were resuspended at 5×10^6 cells/ml and stimulated with 12.5 μg/ml Con A (Amersham Biosciences) for 48 h. CU205 cells were treated with 15 μg/ml tunicamycin (Sigma–Aldrich) for 48 h. The cells were cultured at 41 °C in 5% CO_2.

Poly(A)$^+$ RNA was isolated using the polyA Tract mRNA Isolation System (Promega) from CU205, and Con A-stimulated and normal splenic lymphocytes. The samples were resolved on a 1% formaldehyde agarose gel, transferred to positively charged nylon membrane (Roche Diagnostics, Indianapolis, IN), and hybridized with a digoxigenin-labeled chicken IL-17 cDNA probe in DIG Easy Hyb solution (Roche Diagnostics) at 55 °C overnight. The probe was prepared by PCR using chicken IL-17 specific primers (5′-CAGATGCTGGATGCCTAACC and 5′-CTTTAAGCCTGGTGCTGGAT). The blot was washed twice in 2× SSC and 0.1%

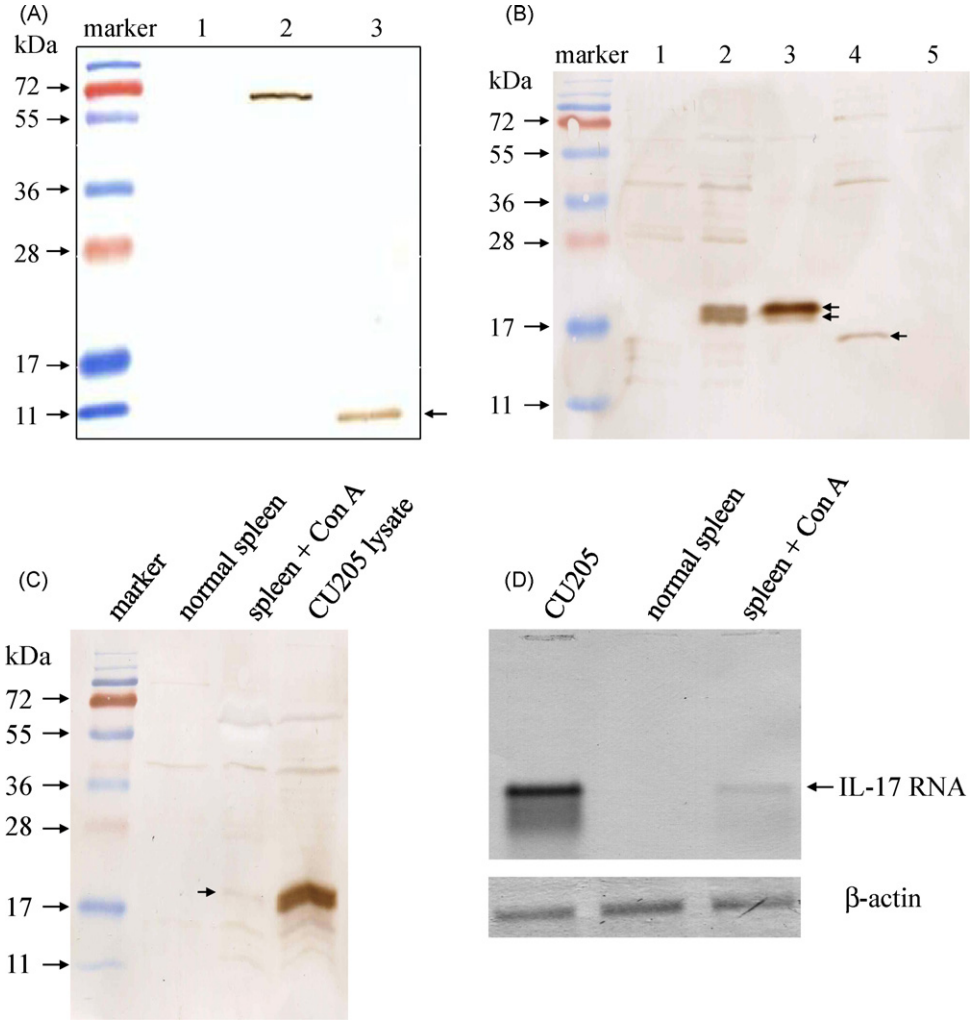

Fig. 2. Western blot analysis of chIL-17 mAb 3D11 and Northern blot analysis of chIL-17 RNA. (A) Bacterially expressed, affinity purified MBP (lane 1) and MBP-chIL-17 (lane 2) were resolved on a SDS-PAGE under reducing conditions. The arrow (lane 3) indicates the recombinant chIL-17 protein after Factor Xa digestion. (B) The cell lysate (lanes 2 and 4) and supernatants (lanes 3 and 5) of CU205 cells cultured in the presence (lanes 4 and 5) or absence (lanes 2 and 3) of 15 μg/ml tuncamycin were run on a SDS-PAGE under reducing conditions. Lane 1 is normal splenic lymphocytes. (C) Con A-treated splenic lymphocytes, normal splenic lymphocytes and the lysate of CU205 cells were resolved on a SDS-PAGE under reducing conditions. The arrows indicate positive bands and the migration of protein size marker is indicated on the left. (D) Northern blot analysis of chIL-17 transcript. Poly(A)$^+$ RNA extracted from CU205 cells and normal and Con A-stimulated splenic lymphocytes were resolved on a 1% formaldehyde agarose gel, transferred to a nylon membrane, and hybridized to a digoxigenin-labeled chIL-17 cDNA probe.

SDS for 15 min at room temperature followed twice by 0.1× SSC and 0.1% SDS for 15 min at 55 °C. Detection was carried out with the DIG High Prime DNA Labeling and Detection Starter Kit (Roche Diagnostics) according to the manufacturer's instruction. Splenic lymphocytes were stimulated with 12.5 μg/ml Con A during 48 h. Chickens were provided free access to feed and water and used for experimentation at 5–7 weeks of age. As a control, the chicken β-actin gene was amplified by PCR with the primers 5′-TCTGGTGGTACCACAATGTACCCT and 5′-CCAGTAATTGGTACCGGCTCCTC.

For intracellular staining of lymphocytes, cells were washed with PBS twice, fixed with 2% paraformaldehyde (Sigma–Aldrich) for 15 min at room temperature, washed twice and permeabilized with PBS including 0.5% saponin (Sigma–Aldrich) for 20 min. Cells were then incubated with undiluted hybridoma culture supernatants for 1 h at room temperature, washed with PBS twice and reacted with FITC-labeled rabbit anti-mouse IgG antibody (Sigma–Aldrich) for 40 min. Cells were washed twice again, resuspended in 0.5% paraformaldehyde and analyzed by flow cytometry (BD Biosciences, USA).

Following initial ELISA screening, six mAbs with high binding activities to chIL-17 were chosen for further characterization. Antibodies 3D11 and 6E8 were determined to be IgG_{2a}, 8B10 and 1G8 were IgG_1, 2A2 was IgG_{2b}, and the antibody 10B8 was IgM. As shown in Fig. 1, the mAb 3D11 specifically recognized MBP-chIL-17 with no significant binding to other proteins (BAS, MBP and DH5α). Other mAbs showed similar results (data not shown).

Given the predicted molecular weight of 43 kDa maltose binding protein (MBP) and 14 kDa chIL-17 (from aa 45 to aa 169), the recombinant fusion protein was estimated to be 57 kDa. By Western blot analysis (Fig. 2A), mAb 3D11 detected the MBP-chIL-17 fusion protein (lane 2), but did not recognize MBP used as a negative control (lane 1). Lane 3 showed the reactivity of mAb 3D11 with the recombinant chIL-17 which is devoid of MBP.

Previously, chIL-17 transcript was highly expressed in CU205 cell line and in the Con A-stimulated splenic lymphocytes indicating high levels of secreted chIL-17 protein in the supernatant of CU205 cells and in the Con A-stimulated splenic lymphocytes (Min and Lillehoj, 2002). As shown in Fig. 2B, the same mAb detected immunoreactive bands of around 20 and 21 kDa in the cell lysate and the concentrated cell-free supernatant of CU205 cells, but not in the normal spleen cell lysate under reducing conditions (Fig. 2B). In humans, two unique proteins with molecular mass of around 15 and 20 kDa were recognized in the supernatant of human IL-17 expressing cells under reducing conditions, indicating that the 15 kDa protein is the backbone of the protein whereas the 20 kDa protein represents an N-linked glycosylated form of the protein (Yao et al., 1995). To investigate whether mAb 3D11 detects the backbone of chIL-17 protein, CU205 cells were treated with tunicamycin to inhibit the addition of N-linked oligosaccharides to glycoprotein. The band of approximately 16 kDa was identified only in the lysate of CU205 cells (Fig. 2B) indicating that mAb 3D11 recognizes the backbone and an N-linked glycosylated form of chIL-17 protein. In the Con A-stimulated splenic lymphocytes, around 20 kDa band was detected also (Fig. 2C).

Northern blot analysis was carried out because Con A-stimulated splenocytes showed faint band by Western blot analysis. As shown in Fig. 2D, IL-17 transcript was detected in Con A-stimulated splenocytes and CU205 cells, but not in normal splenocytes. These results implied that the mAb specifically detected chIL-17 proteins. The remaining five mAbs showed the similar results as 3D11 mAb in Western blot analyses (data not shown).

A crucial step in the production of mAbs using bacterially expressed protein is to provide an evidence for specific detection of the corresponding native form by the reagent. Thus, we performed intracellular staining to test the specific binding of these antibodies to the native chIL-17. As shown in Fig. 3, the binding of lymphocytes with the mAbs 10B8, 2A2 and 1G8 resulted in the shifting of the relative fluorescence intensity peak to the right indicating the recognition of native chIL-17 by these mAbs while the others were negative.

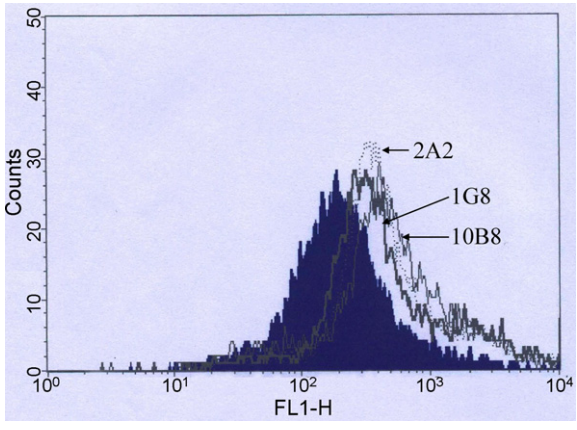

Fig. 3. Intracellular staining of CU205 cells with chIL-17 mAbs. The cells were fixed, stained with mAbs, and analyzed by flow cytometry. Isotype antibody was used as a negative control.

In conclusion, one or more of the mAbs described in this report specifically detected chIL-17 and will be a useful tool for immunological studies of chicken IL-17 in normal and disease states.

Acknowledgments

This research was supported by the MIC, Korea, under the ITRC support program supervised by the IITA (IITA-2006-(C1090-0603-0047)).

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