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A Novel Monoclonal Antibody Against Mouse B7-H3 Developed In Rats

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Abstract

B7-H3, a novel member of the B7 superfamily, plays a critical role during T cell activation and its functions are still unclear. In this study we obtained a novel anti-mouse B7-H3 monoclonal antibody (MAb) and characterized its biological functions. Our results demonstrated that this MAb could be used for flow cytometry, western blot and immunohistochemistry analysis, suggesting that the performance of this MAb is much better than a commercially MAb (M3.2D7). Furthermore, data showed different expression profiles of mouse B7-H3 on various immune cells. We further showed that mouse B7-H3 protein was not expressed on normal tissues except for bladder epithelial cells using this MAb. Interestingly, the MAb could stimulate the proliferation and cytokine secretion of T cells. Taken together, this MAb might be of great value for further investigation of B7-H3 molecule.

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Key words: mouse; B7-H3; monoclonal antibody; T lymphocyte

Introduction

B7 homolog3 (B7-H3), also named CD276, has been identified both in human and mice. Murine B7-H3 gene encodes a 316 amino acid protein which shares about 87% sequence homology with human [1,2]. Mouse B7-H3 protein is a type I transmembrane glycoprotein containing two extracellular Ig domains. Murine B7-H3 mRNA is widely expressed in multiple tissues, but B7-H3 protein is not detected in these tissues [1-3]. Till now B7-H3 receptor has not been identified yet [4,5]. Previous studies showed B7-H3 stimulated the proliferation of T cells and enhanced the secretion of IFN-γ [6]. But subsequent results indicated that B7-H3 down-regulated Th1-mediated immune responses [7,8]. Luo et al. demonstrated that B7-H3 had antitumor activity in mice [9]. Recently, B7-H3 was shown to be uniformly aberrantly expressed in sera or tumor tissues of cancer patients [10-14]. Thus B7-H3 might be a promising target in diagnosis and therapy for malignancies.

In this study, we generated a novel rat anti-mouse B7-H3 MAb and examined its

expression by immunostaining. Furthermore, we found that it could stimulate the proliferation and enhance the cytokine secretion of T cells.

Materials and methods

1 Animals, cell lines, and antibodies

SD Rats were purchased from the Department of Experimental Animal, Shanghai Institute of Biological Products, Ministry of Health of China (Shanghai, China). Mouse myeloma cell line SP2/0, Chinese hamster ovary (CHO) cell and human embryonic kidney cell 293 were originally obtained from American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI 1640 or DMEM (Life Technologies**,** Inc., Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Hyclone, Logan,UT), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 25 mM HEPES buffer. PE-conjugated rat anti-mouse B7-H3 MAb (clone M3.2D7) and PE-conjugated donkey anti-rat IgG (H+L) were purchased from eBioscience (Woburn, MA). HRP-conjugate goat anti-rat IgG (H+L) and rat IgG2b were all purchased from Immunotech (Marseille, France). All chemicals were obtained from Sigma (St.Louis, MO). All immunohistochemistry reagents were obtained from Invitrogen (Carlsbad, CA).

2 Construction of transfectants

The full length cDNA encoding mouse B7-H3 was cloned from bone marrow derived dendritic cells by reverse transcription polymerase chain reaction (RT-PCR) with

specific primers and was inserted into vector pIRES2-EGFP (Clontech, Mountain View, CA). The recombinant vector was transfected into CHO and 293 cells by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The B7-H3 transfected cells (CHO/B7-H3 and 293/B7-H3) were selected by G418 (Invitrogen) and confirmed by a combination of commercial PE-conjugated mouse B7-H3 MAb (M3.2D7) and GFP using flow cytometry (Beckman Coulter, Brea, CA). Empty vector-transfected CHO and 293 cell lines (CHO/mock and 293/mock, respectively) were obtained in the same way.

3 Generation of anti-mouse B7-H3 MAb

Female SD rats were immunized with injections of 1×10^7 293/B7-H3 cells in 0.5 ml phosphate-buffered saline (PBS) per rat at 21 day intervals for a total of four times. The first subcutaneous injection was accompanied with complete Freund's adjuvant. Four days after the final boost injection, the splenocytes of immunized rats were fused with murine myeloma SP2/0 cells according to the method described previously [15]. Flow cytometry (Beckman Coulter) was performed to screen positive clones. CHO/B7-H3 cells were used as the positive control and CHO/mock cells were used as the negative control. The anti-mouse B7-H3 MAb was purified from the ascites of nude mice using protein G-sepharose CL4B affinity columns (Pharmacia, Uppsala, Sweden).

4 Characterization of MAb

The Ig isotype was identified with multiplex fluorescent bead assay (SouthernBiotech, Birmingham, USA) according to the manufacturer's instructions. To analyze the

expression of mouse B7-H3 molecule on cells including T cells, DCs, monocytes, NK and B cells, 1×10^6 cells were incubated with MAb 18F9 for 30 min at 4 ℃. After washing with PBS, the cells were stained with PE-conjugated donkey anti-rat IgG for another 30 min, and analyzed using flow cytometry. Western blotting was performed to analyze the binding capacity of the two MAbs (18F9 and M3.2D7) to recombinant B7-H3-Ig. Briefly, 5μg of purified B7-H3-Ig was mixed with loading buffer and boiled at 95-100□ for 5 min followed by separation on 10% SDS-polyacrylamide gels, transferred onto a nitrocellulose membrane which was incubated with biotinylated anti-mouse B7-H3 MAbs or Rat IgG2b isotype control for 1h. After washing, Then the membrane was stained with horseradish peroxidase (HRP)-conjugated goat anti-rat IgG for 2 h. Results were observed utilizing the Chemiluminescence Western Blotting Kit from Boehringer (Mannheim, Germany) according to the manufacturer's instructions.

5 Immunohistochemical staining

The paraffin sections of mouse tissues were collected for immunohistochemical staining. In brief, after dewaxing, incubate sections with 3% H_2O_2 in PBS for 10 minutes to block endogenous peroxidase. Sections were treated with EDTA working dilution for antigen retrieval. After cross-reactivity was blocked with normal non-immune rat serum, the sections were incubated at 37℃ for 1 h with biotinylated MAb 18F9 (10 μg/ml). The sections were incubated with streptavidin-HRP for 30 minutes, then DAB solution for 3 minutes. Negative controls were established by

replacing the primary antibody (MAb 18G9) with rat IgG2b isotype control. Positive staining of B7-H3 was defined as the presence of brown color in cytoplasm and on cell membrane.

6 Cell proliferation assay

T cells were cultured in 96-well cell culture plate in triplicate (1×10^5 cells/well) in complete RPMI 1640 at 37□, 5% CO_2. The wells were coated with agonistic anti-CD3 MAb (0.5 μg/mL). T cells were incubated with various concentration of MAb 18F9 for up to 3 days. Cell proliferation was analyzed by cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) at 2, 6, 12, 24, 48, and72 h, respectively. The cytokine levels in the supernatants of T cell cultures were assessed by the following commercial ELISA kits: IFN-γ and IL-2 (Invitrogen/Biosource, Carlsbad, USA) according to the manufacturer's instructions.

Statistics analysis

The statistical analysis was performed using two-tailed t-test, and a *p* value less than 0.05 was considered significant.

Results

Establishment of a novel rat anti-mouse B7-H3 MAb

We cloned full length of mouse B7-H3 cDNA from mouse DCs and transfected it into 293 cells and CHO cells, respectively. To generate MAbs specific for mouse B7-H3,

the splenocytes of rats immunized with 293/B7-H3 cells were fused with myeloma SP2/0 cells. After few cycles of screenings and sub-clonings, one stable hybridoma cell line named 18F9 was obtained (Fig. 1A). As shown in Figure 1B, this MAb could recognize transfected cells, CHO/B7-H3 and 293/B7-H3, but not CHO/mock, CHO/TLT2, 293/mock and 293/B7-H4 cells, indicating that the MAb is specific for B7-H3. Western blotting was performed to confirm the binding ability of 18F9 to recombinant B7-H3-Ig. The data in Figure 1C showed that, unlike commercially MAb M3.2D7, 18F9 specifically bound to B7-H3-Ig. Thus we prepared one specific rat anti-mouse MAb which could be used for flow cytometry and western blotting analyses. The isotype of the MAb was IgG2b subclass with a κ light chain (Fig.1D).

Expression of mouse B7-H3 molecule by immune cells

The expression of mouse B7-H3 was examined on immune cells by flow cytometry analysis. In spleen, B7-H3 was not expressed on NK cells, resting and activated T cells (Fig.2A and 2B). In contrast, 29.02% of $B220^+$ B cells (Fig.2C) and 82.88% of $CD14^+$ monocytes (Fig.2D) constitutively expressed B7-H3. Bone marrow-derived immature and mature dendritic cells both expressed B7-H3 (Fig.2E and 2F) suggesting that mouse B7-H3 was expressed on antigen presenting cells (APCs).

MAb 18F9 recognizes B7-H3 molecule in tissues

The expression of Murine B7-H3 on various tissues was investigated by immunohistochemistry staining with MAb 18F9. Results showed that murine B7-H3 protein was not expressed in normal tissues except for bladder epithelial cells (Fig.3).

Thus, MAb 18F9 performed well in immunohistochemical stainings and may have great value for the study of bladder cancers.

Effect of mAb 18F9 on T cell proliferation

In this study, the biological effect of MAb 18F9 on T cell proliferation and cytokine production *in vitro* was performed by assays of CCK8 and ELISA. Interestingly, we found that MAb 18F9 could obviously promote the proliferation of T cell at a dose-dependent manner (Fig.4A). The secretion of cytokines (IL-2 and IFN-γ) reached maximum levels at 24h stimulated by MAb 18F9 (Fig.4B̃)

Discussion

B7-H3, a sixth member of the B7 ligand family, was first identified as a positive co-stimulatory regulator of T cell responses [6]. However, the regulatory roles of B7-H3 is controversial after further investigation. Both positive and negative effects of B7-H3 during immune response have been reported [6-8]. Mouse B7-H3 mRNA was expressed in multiple tissues while the expression profile of B7-H3 protein is not clear. B7-H3 has a soluble form and its level was increased in the sera of lung cancer patients as well as other diseases [16-18]. But the physiologic and pathologic roles of B7-H3 are little understood. In order to explore the functions of B7-H3 molecule, one specific rat anti-mouse MAb 18F9 was prepared. Titration studies showed that 18F9 could recognize a different epitope of B7-H3 from M3.2D7 (data not shown). Our further studies found that this MAb could be used in western blotting analysis,

immunocytostaining and immunohistochemical staining. We also analyzed the expression of the MAb by immune cells. The results showed that mouse B7-H3 molecule was expressed on B cells, monocytes, immature and mature dendritic cells, but not on NK cells, resting and activated T cells. We also found the MAb we generated could promote T cells proliferation and the secretion of IL-2 and IFN-γ. Murine B7-H3 protein was expressed only on the cytoplasma and membrane of bladder epithelial cells instead of any other tissues we investigated. In conclusion, our studies provide a novel and useful tool to study the expression and functions of mouse B7-H3 for both basic and applied purposes.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest and the generated antibody was distributed solely to nonprofit research organizations for research purposes only.

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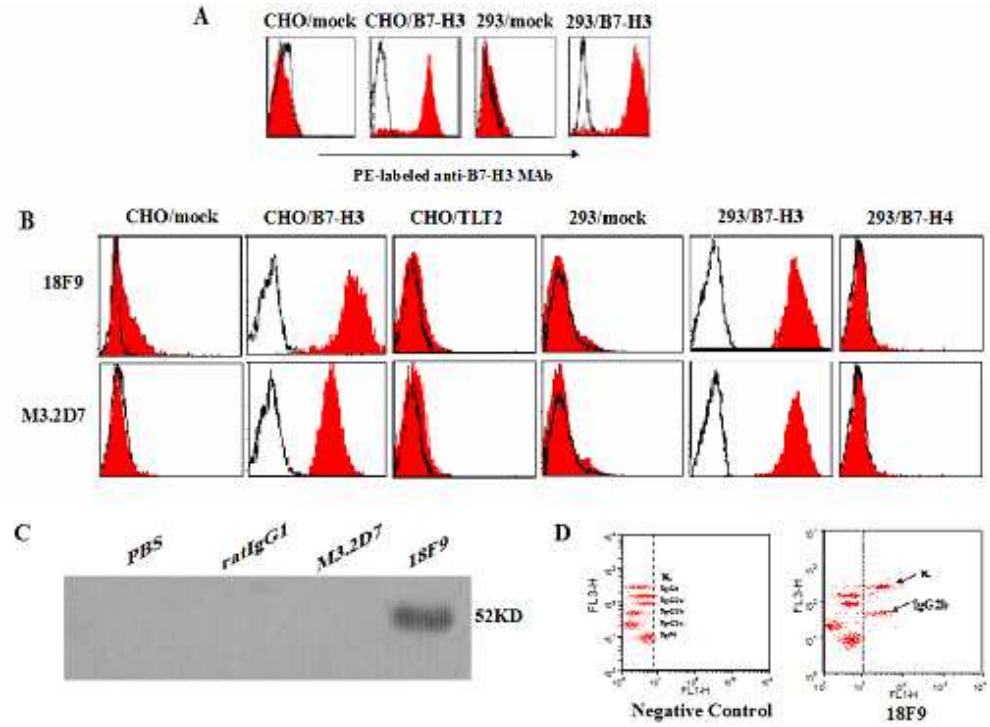
Fig.1. Preparation of mouse B7-H3 transfectants and characterization of B7-H3 MAb. (A) CHO/B7-H3 and 293/B7-H3 transfectants were selected and identified by flow cytometry using PE-labeled M3.2D7 (red histograms) against PE-conjugared rat IgG2b (open histograms). (B) Reactivity of the two MAbs with the transfectants CHO/mock, CHO/B7-H3, CHO/TLT2, 293/mock, 293/B7-H3 and 293/B7-H4. Cells were stained with MAbs (18F9 and M3.2D7) (red histograms) or negative control rat IgG2b (open histograms). (C) Purified B7-H3-Ig was separated on 10% SDS-polyacrylamide gels, transferred to NC membrane and stained with PBS, rat IgG2b, M3.2D7 and 18F9. (D) The Ig isotype of the MAb was identified with carboxyl blue-based bead assay. Negative control and 18F9 staining were shown in left figure (without hybridoma supernatant) and right figure (with hybridoma supernatant), respectively. The figure is a representetive of four independent experiments.

Fig.2. Expression of mouse B7-H3 on immune cells. The Expression of B7-H3 by spleen cells (A, B, C and D) and bone marrow-derived dendritic cells (E and F) were examined by 18F9 in conjunction with other indicated markers. Rat IgG2b was used as an isotype control. The figure is a representetive of three independent experiments.

Fig.3. Immunohistochemistry analysis of B7-H3 in mouse tissues by MAb 18F9. Heart (A×40), muscle (B×40), liver (C×100) and bladder (D×40). Brown color was defined as positive staining. All these experiments used rat IgG2b as an isotype

control. The figure is a representetive of three independent experiments.

Fig.4. T cell proliferation and cytokine production stimulated by MAb 18F9. Purified T cells were activated with plate-bound agonistic anti-CD3 MAb. The MAb 18F9 was applied and cell proliferation (A) and cytokines production (B) were measured. Rat IgG2b was used as an isotype control. *$p < 0.05$ The data shown are representetive of four independent experiments with similar results.



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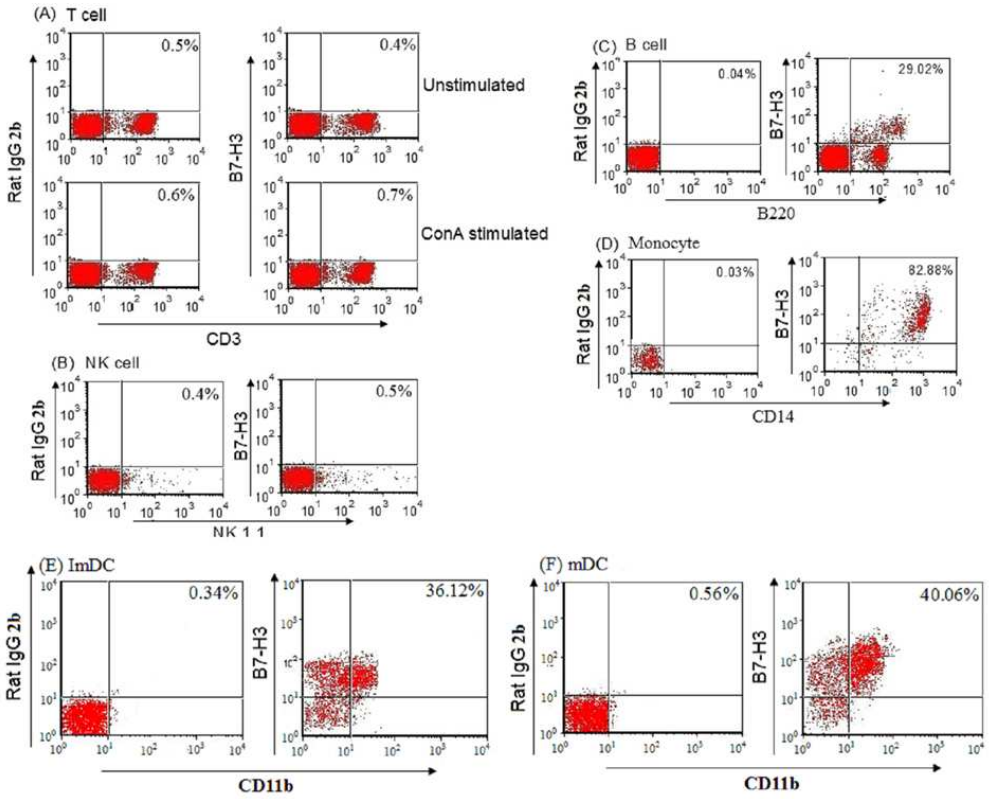

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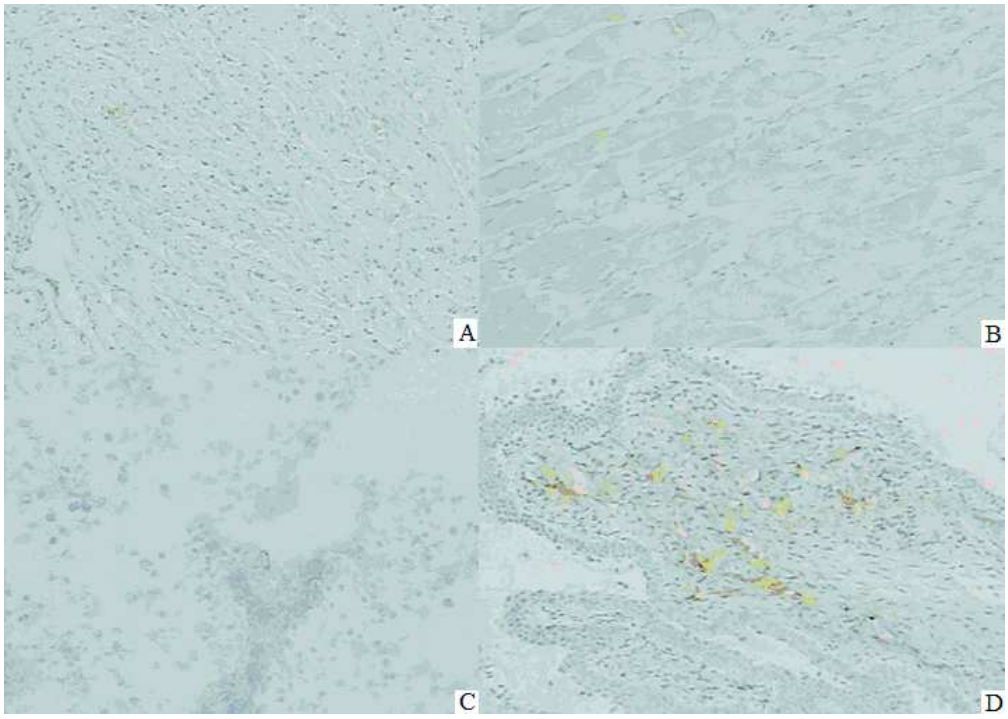

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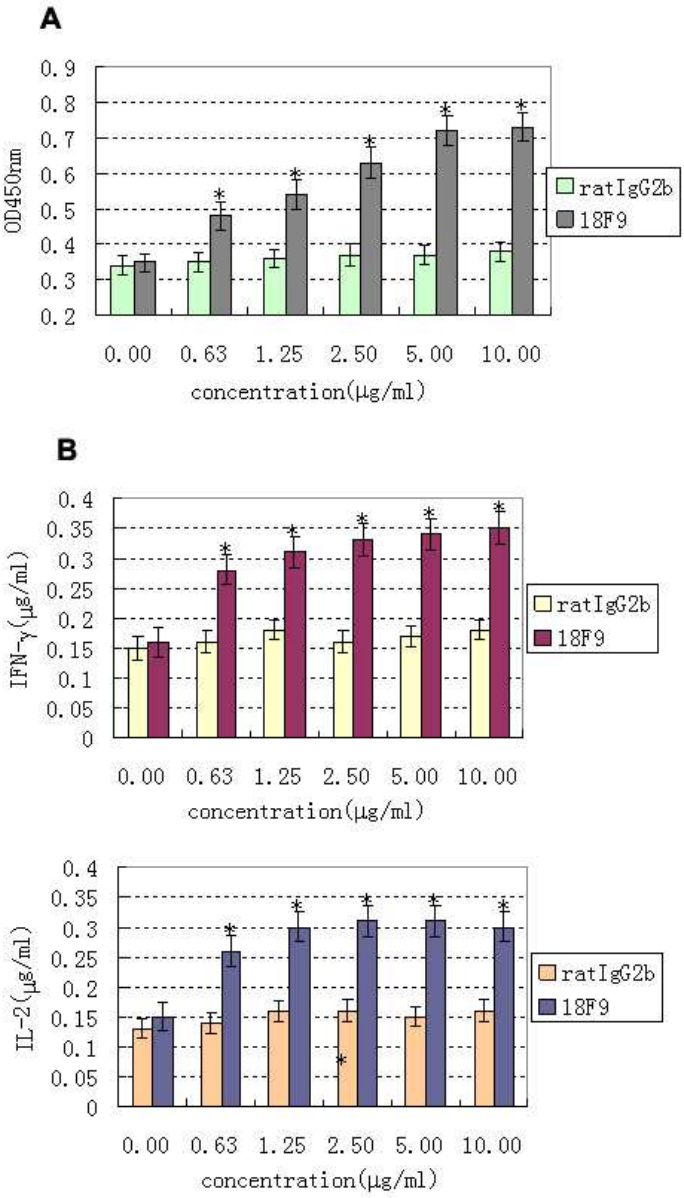

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