Article

B7-H3: Another Molecule Marker for Mo-DCs?

Guangbo Zhang[1, 2], Qiuming Dong[2], Ying Xu[2], Gehua Yu[2] and Xueguang Zhang[1, 2, 3]

Using a newly generated monoclonal antibody (2E6) against human B7-H3, we explored the expression of the molecule on dendritic cells derived from monocytes (Mo-DCs). Its expression was examined by means of immunostaining and flow cytometric (FCM) analysis. The results showed that B7-H3 was expressed in the course of Mo-DC maturation induced with interleukin 4 (IL-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF). The expression could be detected at all the stages of Mo-DC differentiation, and remained at a quite stable level. Interestingly, B7-H3 was not expressed by T cells and B cells, even these cells were activated respectively by PHA or PWM. A weak expression could be detected on resting monocytes. These data showed that constitutive expression of B7-H3 at a high level was found on imDCs and mDCs derived from monocytes. Due to no expression on T cells and B cells, we speculate that B7-H3 might be another valuable molecule marker for Mo-DCs. ***Cellular & Molecular Immunology*****. 2005;2(4):307-311.**

Key Words: B7-H3, costimulatory molecule, Mo-DC, monoclonal antibody

Introduction

B7-H3, a B7-like molecule, was recently identified upon database searches for sequences with homologies to B7 molecules (1). Like most other members of this family, B7-H3 was described as a type I membrane protein with two Ig-like domains (2IgB7-H3). Human and mouse B7-H3 shares ~87% amino acid sequence homology. Following that, two different groups reported the isoform of human B7-H3 gene with four Ig-like domains (B7-H3b or 4IgB7-H3) (2, 3). Similar to other B7 homologs, mRNA of B7-H3 was detected on a broad spectrum of tissues even in some malignant tumor cell lines, such as chronic myelogenous leukemia K562 cells, lung carcinoma A549 cells (1). In one of our previous studies, the expression could be detected on DCs and some tumor cells (4). The receptor for B7-H3 was unknown until now, but the counter-receptor that is distinct from CD28, CTLA-4, ICOS, and PD-1 was observed to be rapidly induced following T lymphocyte activation (1). B7-H3 was first reported as a positive costimulatory molecule to stimulate the proliferation of T cells and selectively enhanced IFN-γ secretion with modest effects on TNF-α (1). But the following experimental data indicated that the biological functions of B7-H3 were inconsistency. Analyzing the B7-H3-deficient mice, Suh et al. reported B7-H3 down-regulated immune responses mediated by Th 1 and inhibited the secretion of cytokine IFN-γ (5). Sun et al. found B7-H3 could enhance antitumor immunity in mice challenged with EL-4 cells transfected to express B7-H3 (6). In our previous study, it was also found that the recombinant protein GST/hB7-H3 expressed in *E. coli* could induce T cell proliferation and IL-10 production (7).

The aim of the present study was to analyze the expression of B7-H3 on Mo-DCs by a newly generated monoclonal antibody against B7-H3 in our laboratory. Compared with the observation of the induced expression of B7-H3 on DCs, we found the molecule was constitutive expressed. Therefore, we thought B7-H3 could be identified as a valuable surface antigen for Mo-DCs.

Materials and Methods

Antibody and cell culture

The cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (FCS, Hyclone, USA) with 2 mM L-glutamine and 100 U/ml penicillin-streptomycin. Cells were cultured in 5% CO_2, at 37˚C. The series of anti-human mAbs including CD3, CD11c, CD14, CD19, CD83 and mouse/human IgG protein were all purchased from Immunotech Company (France), if not specially pointed out. The trasfected cell line B7-H3/L929 and the mAbs (4H7 and 21D4) against B7-H3 were prepared and kept by our laboratory.

[1]Clinical Immunology Laboratory of Jiangsu Province, Suzhou University No.1 Affiliated Hospital, 96 Shizi Street, Suzhou 215006, China;

[2]Biotechnology Institute, Suzhou University, 48 Renmin Road, Suzhou 215007, China;

[3]Corresponding to: Dr. Xueguang Zhang, Biotechnology Institute, Suzhou University, 48 Renmin Road, Suzhou 215007, China. Tel: +86-512-651-25011, Fax: +86-512-651-04908, E-mail: smbxuegz@public1.sz.js.cn.

Received Jul 30, 2005. Accepted Aug 23, 2005.

Production of anti-B7-H3 mAb

The Balb/c mice were immunized with injections of 1×10^7 mitomycin-treated 293T cells expressing B7-H3 (4, 8) in 0.5 ml of phosphate buffered saline (PBS) per mouse for four times. The splenocytes of the immunized mice were fused with SP2/0 cells according to the previous method (4). And hybridoma cell lines were screened by flow cytometry with B7-H3/L929 transfectants and/or 293T cells as positive cells, Vector/L929 transfectants as negative cells sequentially. The mAb was purified from ascites of Balb/c mouse using protein G affinity column (Amersham pharmacia Inc., Sweden).

Preparation and stimulation of T cells, B cells and monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human peripheral blood (Suzhou Central Blood Bank, China) by Ficoll-Hypaque (Shanghai Second Chemistry Factory, China) gradient centrifugation. Monocytes were prepared from PBMCs by removing nonadherent cells after 2 h incubation in six-well culture plates in RPMI 1640 with 10% FCS at 37°C, and then stimulated by LPS for 24 h. The nonadherent cells including T, B cells were cultured in RPMI 1640 containing 10% FCS and stimulated by PHA (1 μg/ml), pokeweed mitogen (PWM) at 4 mg/L for 24 h.

Isolation and culture of Mo-DCs

The monocytes were prepared as above and cultured in RPMI 1640 containing 10% FCS, 100 ng/ml of rhGM-CSF (R&D) and 50 ng/ml of recombinant human IL-4 (R&D). For the stimulation of monocytes, the anti-CD40 mAb 5C11 (product of our laboratory, 10 μg/ml) (9) or TNF-α (Sigma; 10 ng/ml) was added to the culture. Cultures were fed with fresh, cytokine-containing medium, and the morphology of cells was monitored daily by light microscopy.

Western blotting

Western blotting was performed under standard conditions using anti-B7-H3 mAb 2E6 (1 μg/ml) according to the previous paper (4). Bound Abs were detected using AP-conjugated goat anti-mouse Ig Abs (DAKO, alostrup, penmark; 1/1000). After briefly rinsed with washing buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$), the specific protein bands were visualized by BCIP/NBT (Watson Biotech, Shanghai, China).

Flow cytometric analysis

For flow cytometric analysis, cells (1×10^7/ml) were incubated with the mAb against B7-H3 for 30 min on ice and washed. For indirect staining, PE- or FITC-labeled goat anti-mouse antibody as secondary antibody was incubated for another 30 min on ice and washed. The results were analyzed by Flow Cytometry Facility (Beckman-Coulter, Altra, USA) and the Beckman-Coulters Expo32 MultiComp software.

Results

Establishment of a specific mouse anti-human B7-H3 mAb

To prepare the mAb against human B7-H3, we used the cell line 293T expressing B7-H3 as an immunogen. After several fusions and subclonings, one stable hybridoma cell line named as 2E6, secreting specific antibody against human B7-H3 was obtained. Immunophenotyping indicated that the mAb recognized human B7-H3 expressed on B7-H3-transfected L929 cells and 293T cells (Figure 1A). In further analysis, we found the B7-H3Ig fusion protein (prepared by our laboratory) could block 2E6 to recognize B7-H3 on the transfected cells, which confirmed the mAb was special against B7-H3 (Figure 1B). The results of competitive inhibition test indicated that the mAb recognized a different epitope from mAb 4H7 or 21D4 established previously in our laboratory (Figure 1C). The result of Western blotting further confirmed that the newly generated mAb 2E6 was specific against B7-H3 (Figure 2).

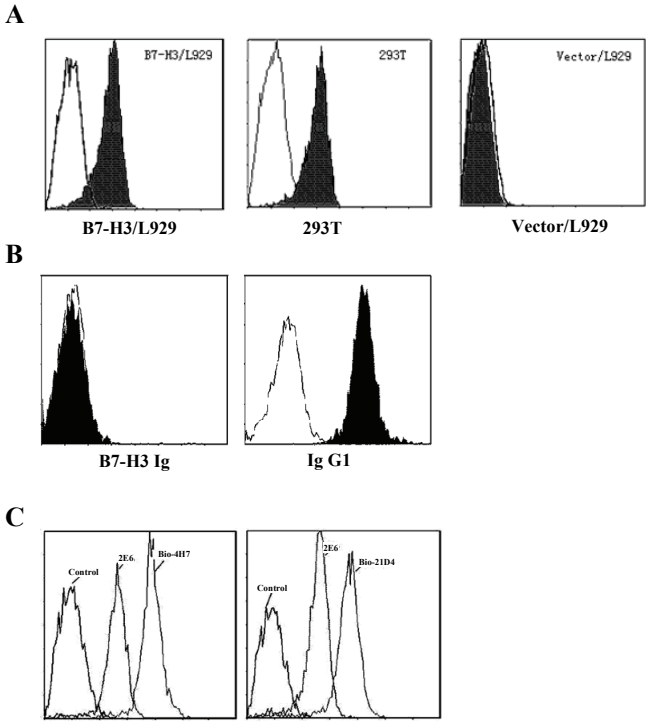

Figure 1. Identification of the mAb 2E6 against B7-H3. (A) Specificity of mAb 2E6 (filled) against B7-H3 was determined by flow cytometric analysis using 293T cells expressing B7-H3, B7-H3/L929 transfected cells and Vector-transfected L929 cells. Mouse IgG was used as negative control (open). (B) The mAb 2E6 (filled) was also tested for competitive binding in the presence of 10 μg of hB7-H3Ig or hIgG. The result showed hB7-H3Ig fusion protein could block the binding of 2E6 and B7-H3 expressed on the transfected cells, which proved that the mAb 2E6 was special against B7-H3. Mouse IgG was used as negative control (open). (C) The competitive inhibition test, after 293T cells were incubated with 2E6 for 30 min follwed by FITC-labled goat anti-mouse IgG, the cells were respectively stained with biotinylated anti-B7-H3 mAb 4H7-biotin or 21D4-biotin followed by streptavidin-PE. The results showed the mAb 2E6 recognized a novel epitope from that of the mAb 4H7 or 21D4 recognized. Mouse IgG was used as negative control.

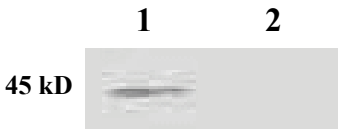

Figure 2. Identification of anti-B7-H3 mAb 2E6 with Western blotting. The mAb 2E6 recognizes a protein of ~45 kD which was in agreement with the molecular weight reported in the previous paper (4). Lane 1, B7-H3/L929 cells; Lane 2, Vector-L929 cells.

MAb 2E6 specifically recognizes its cognate antigen on different cell types

We first examined the expression of B7-H3 on T cells, B cells and monocytes by flow cytometric analysis using the mAb 2E6. As shown in Figure 3, T cells and B cells, as well as T and B cells activated by PHA or PWM respectively did not express B7-H3. A weak expression could be detected on freshly isolated monocytes, but when stimulated by LPS, the expression of B7-H3 could be significantly upregulated.

Expression of B7-H3 on Mo-DCs induced with cytokines in vitro

Monocytes were cultured in six-well plates in fresh complete medium supplemented with 1,000 U/ml GM-CSF and 500 U/ml IL-4. To generate mature Mo-DCs, immature DCs were subsequently stimulated with 5C11 or recombinant human TNF-α for 48 h (Figure 4A). The expression of B7-H3 was detected every 48 h. As shown in Figure 4B, stable expression of B7-H3 was found till day 6 of the culture. After

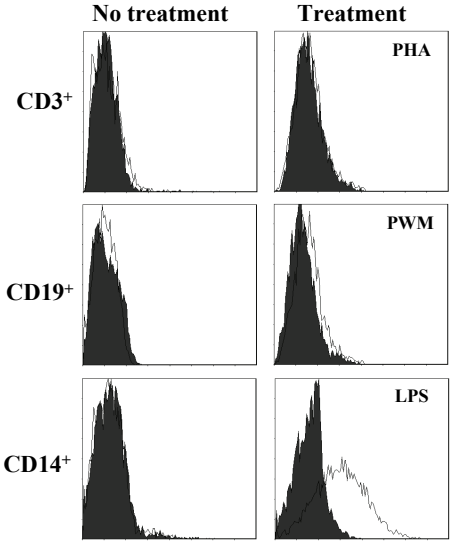

Figure 3. Reactivity of mAb 2E6 with freshly isolated and *in vitro*-stimulated lymphocytes. *In vitro*-stimulated PBLs were stained with FITC-conjugated 2E6 mAb (open). The shaded histograms indicate background staining with control mouse IgG. The figure was representative of at least three experiments.

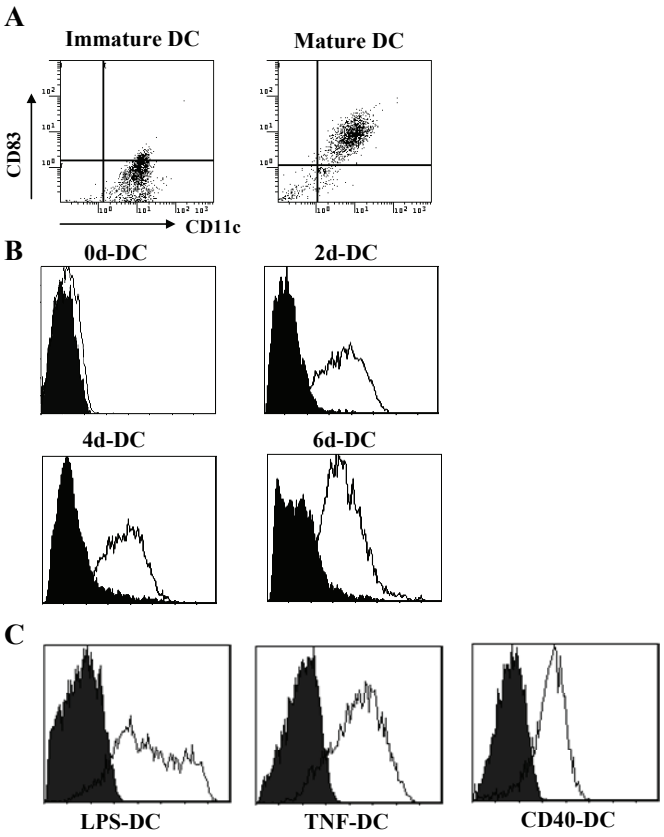

Figure 4. Detection of B7-H3 expression on Mo-DCs. (A) The immature Mo-DCs and mature Mo-DCs. (B) The freshly isolated monocytes were cultured with IL-4 and GM-CSF. The expression of B7-H3 remained at a quite stable level on day 2, 4, 6. (C) After 6 days of culture, the cells were stimulated to maturation with TNF-α, anti-CD40 mAb or LPS. B7-H3 expression (open) still sustained a stable high level. The shaded histograms indicated background staining with control mouse IgG. The figure was representative of three experiments.

that, the imDCs were stimulated with different cytokines or antibody for another 2 days, the expression of the molecule was detected by FCM. The expression still remained at a high level (Figure 4C). Compared with the surface molecules on Mo-DCs such as CD83, HLA-DR and CD14, the expression of B7-H3 on Mo-DCs was more stable (Figure 5).

Discussion

An optimal T cell response requires two types of signals. One is delivered by the TCR upon recognition of specific peptide-MHC complexes displayed on the surface of APCs. The second signal is provided by costimulatory or coinhibitory receptors that bind to their cognate ligand costimulatory molecules (CMs) on APCs (10). The most intensively characterized CMs were those of the B7 family. The classical members B7-1 (CD80) and B7-2 (CD86) of this family interacted with CD28 on naïve T cells, and stimulated IL-2

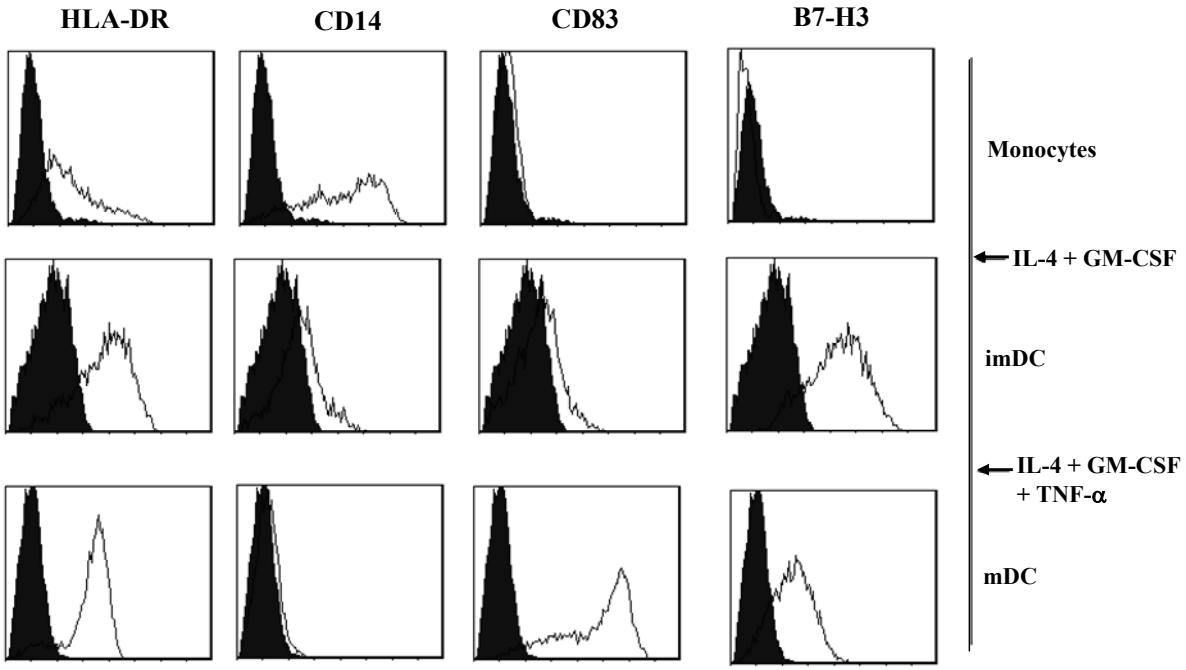

Figure 5. Comparison of the expression of surface molecules on Mo-DCs. At the stages of Mo-DC differentiation and maturation, the surface molecules such as CD14, HLA-DR, CD83 and B7-H3 were detected by FACS. The shaded histograms indicated background staining with control mouse IgG.

production (11). B7-1 and B7-2 also bind CTLA4 (CD152), a homologue of CD28 that inhibits T cells counterbalancing CD28 (12). B7-1 and B7-2 bind CTLA4 with higher affinity than CD28 (13). Recently, the several new members (B7-H1, B7-H2, B7-H3, and B7-DC) of the B7 family were identified; the expression and their structures and functions were elucidated. It was proposed that they regulated the function and differentiation of effector lymphocytes in the periphery. The B7-H1 (PD-L1) and B7-DC (PD-L2) bound to the receptor PD-1 on T cells, inhibited T cell proliferation and cytokine production (14-17). The expression of PD-L1 or B7-H1 on tumor cells of a variety of histologies has suggested a potential mechanism for tumor escape from immune destruction (18-20). The B7-H2 (B7h, the B7-related protein1, GL50, LICOS) bound to inducible costimulator (ICOS) on T cells, appeared to play a major role in regulating Th2 responses (21-23). B7-H4 (B7x, B7s), the engaging B7 family member, was originally observed to interact with BTLA (B and T lymphocyte attenuator) to down-regulate the T-mediated immune response (24-26), but latest research revealed that B7-H4 is not a ligand for BTLA (27).

The expression and biofunctions of B7-H3, which is the new member of the B7 family, have not been elucidated. In this study, we used the 293T cells expressing B7-H3 as immunogen preparing one novel monoclonal antibodies against B7-H3 which recognized different epitope from the previous two anti-B7-H3 mAbs 4H7 and 21D4 obtained by our laboratory. Our data showed that B7-H3 was expressed constitutively at a high level on Mo-DCs. Additionally, LPS could rapidly induce high-level expression of B7-H3 on monocytes. But, the expression of B7-H3 could not be detected on resting or activated T and B cells. These results suggested that B7-H3 might be an important antigen for Mo-DCs by using our newly generated anti-B7-H3 mAb 2E6.

Chapoval et al. reported that IFN-γ was needed for stimulating the expression of B7H3 on DCs (1), but we found B7-H3 was constitutively expressed on both immature and mature Mo-DCs. The expression on Mo-DCs maintained steadily high during the process of Mo-DC maturation. Therefore, we speculated B7-H3 might be another molecular marker of Mo-DCs.

In fact, there are no "specific" markers for DCs including CD1a, which is in fact a cortical thymocyte marker that is also expressed on some types of DCs. DC populations can be separated by FACS by their "lineage negative" characterisation and high expression of HLA-DR. Sub-populations of blood DCs express CD11c, while activated or mature blood DCs express costimulatory molecules such as CD83 and CD86.

In summary, one novel mAb recognizing B7-H3 was successfully generated. Using the mAb, we demonstrated that human B7-H3 could be expressed constitutively on Mo-DCs, which might be a valuable molecule to characterize Mo-DCs. The biological functions of B7-H3 in immune responses remain unclear and the receptor of B7-H3 is unknown. However, it is conceivable that the expression of B7-H3 on Mo-DCs could affect the immune regulatory system under certain conditions, and the biofunction remains to be

elucidated in the further study.

Acknowledgement

This work is supported by a grant from the National Natural Science Foundation of China (No.30330540).

References

1. Chapoval AI, Ni J, Lau JS, et al. B7-H3: a costimulatory molecule for T cell activation and IFN-γ production. Nat Immunol. 2001; 2:269-274.
2. Sun M, Richards S, Prasad DV, Mai XM, Rudensky A, Dong C. Characterization of mouse and human B7-H3 genes. J Immunol. 2002;168:6294-6297.
3. Steinberger P, Majidic O, Derdar SV, et al. Molecular characterization of human 4Ig-B7-H3, a member of B7 family with four Ig-like domains. J Immunol. 2004;172:2352-2359.
4. Zhang GB, Zhou H, Chen YJ, et al. Characterization and application of two novel monoclonal antibodies against 2IgB7-H3: expression analysis of 2IgB7-H3 on dendritic cells and tumor cells. Tissue Antigens. 2005;66:83-92.
5. Suh WK, Gajewska BU, Okada H, et al. The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. Nat Immunol. 2003;4:899-906.
6. Sun X, Vale M, Leung E, et al. Mouse B7-H3 induces antitumor immunity. Gene Therapy. 2003;10:1728-1734.
7. Zhang GB, Chen YJ, Shi Q, et al. Human recombinant B7-H3 expressed in *E. coli* enhances T lymphocyte proliferation and IL-10 secretion *in vitro*. Acta Biochimica Biophysica Sin. 2004;36:430-436.
8. Roberta C, Alessandra D, Raffaella A, et al. Identification of 4Ig-B7-H3 as a neuroblastoma-associated molecule that exerts a protective role from an NK cell-mediated lysis. Proc Natl Acad Sci U S A. 2004;101:12640-12645.
9. Zhou ZH, Wang JF, Wang YD, et al. An agonist anti-human CD40 monoclonal antibody that induces dendritic cell formation and maturation and inhibits proliferation of a myeloma cell line. Hybridoma. 1999;18:471-478.
10. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. Annu Rev Immunol. 1996;14:233-258.
11. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. Nat Rev Immunol. 2002;2:116-126.
12. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA. CTLA-4 is a second receptor for the B cell activation antigen B7. J Exp Med. 1991;174:561-569.
13. Linsley PS, Greene JL, Brady W, Bajorath J, Ledbetter JA, Peach R. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA4 receptors. Immunity. 1994;1:793-801.
14. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, costimulates T-cell proliferation and secretion of interleukin-10. Nat Med.1999;5:1365-1369.
15. Latchman Y, Wood CR, Chernova T, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol. 2001;2:261-269.
16. Tamura H, Dong H, Zhu G, et al. B7-H1 costimulation preferentially enhances CD28-independent T-helper cell function. Blood. 2001;97:1809-1816.
17. Wang S, Bajorath J, Flies DB, Dong H, Honjo T, Chen L. Molecular modeling and functional mapping of B7-H1 and B7-DC uncouple costimulatory function from PD-1 interaction. J Exp Med. 2003;197:1083-1091.
18. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med. 2002;8:793-800.
19. Curiel TJ, Wei S, Dong H, et al. Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. Nat Med. 2003;9:562-567.
20. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc Natl Acad Sci U S A. 2002;99:12293-12297.
21. Wang S, Zhu G, Chapoval AI, et al. Costimulation of T cells by B7-H2, a B7-like molecule that binds ICOS. Blood. 2000;96: 2808-2813.
22. Yoshinaga SK, Whoriskey JS, Khare SD, et al. T-cell co-stimulation through B7RP-1 and ICOS. Nature. 1999;402:827-832.
23. Swallow MM, Wallin JJ, Sha WC. B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNF-α. Immunity. 1999;11:423-432.
24. Sica GL, Choi IH, Zhu G, et al. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. Immunity. 2003; 18:849-861.
25. Prasad DV, Richards S, Mai XM, Dong C. B7S1, a novel B7 family member that negatively regulates T cell activation. Immunity. 2003;18:863-873.
26. Watanabe N, Gavrieli M, Sedy JR, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nat Immunol. 2003;4:670-679.
27. Sedy JR, Gavrieli M, Potter KG, et al. B and T lymphocyte attenuator regulates T cell activation with herpesvirus entry mediator. Nat Immunol. 2005;6:90-98.