Article

Soluble Mouse B7-H3 Down-Regulates Dendritic Cell Stimulatory Capacity to Allogenic T Cell Proliferation and Production of IL-2 and IFN-γ

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B7-H3 is a recently identified member of the B7 gene family. Its ubiquitous expression in both lymphoid and nonlymphoid tissues suggests that it could play an important role in the maintenance of self-tolerance. However, the exact function of B7-H3 is still elusive. The purpose of current study is to demonstrate the possible function of soluble mouse B7-H3 for prevention of DC-mediated T cell activation. For this purpose, we established a soluble mouse B7-H3 fusion protein (mB7h3-hIg) eukaryotic expression vector (pmB7h3-hIg) with a C-terminal human IgG1 Fc. A C57BL/6 (B6)-derived dendritic cell line (DC2.4 cells) was used for the establishment of stable transfectants for generation of soluble mB7h3-hIg. Ectopic mB7h3-hIg expression was confirmed by RT-PCR, Western blot and ELISA analyses. A 49.7 kD protein was detected by Western blot from DC2.4 cells transfected with pmB7h3-hIg. It was found that soluble mB7h3-hIg expression has no effect on cell cycling and apoptosis and the expression of CD80 and CD86 of the DC2.4 cells. However, ectopic soluble mB7h3-hIg expression was found to significantly affect the allo-stimulatory capability for DC2.4 cells. DC2.4 cells expressing soluble mB7h3-hIg showed a significant reduced allo-stimulatory capability as compared with the controls determined by MLC. Further studies revealed that soluble mB7h3-hIg could also inhibit IL-2 and IFN-γ production of allogenic T cells. These results suggested a great potential of soluble B7-H3 for treatment of graft rejection and autoimmume disease. ***Cellular & Molecular Immunology*****. 2006;3(3):235-240.**

Key Words: B7-H3, dendritic cell, T cell, proliferation, IL-2, IFN-γ

Introduction

B7 family belongs to the immunoglobulin (Ig) superfamily, among which, B7-1 (CD80) and B7-2 (CD86) are the most extensively studied co-stimulatory molecules. Interaction of CD28 on T cells with either B7-1 or B7-2 has been found to augment T cell activation, promote T cell survival and enhance IL-2 production (1-3). In contrast, interaction of B7-1 or B7-2 with cytotoxic T lymphocyte antigen 4 (CTLA-4), a homolog of CD28, may inhibit T cell response by delivering a negative signal (4, 5). Recent studies have characterized additional members for the B7 family (e.g., B7-H1, B7-H2, B7-DC, B7-H3, B7-H4, BT3) and CD28 family (e.g., PD-1, BTLA) (6-9). Functional study of these new members for B7 and CD28 family indicated that they played an important role in T cell activation.

B7-H3 is a B7 homolog. The mRNA of human B7-H3 (hB7-H3) was detected on a broad spectrum of tissues even in some malignant tumor cell lines, such as the chronic myelogenous leukemia K562 cells and the lung carcinoma A546 cells. However, it was undetectable in the resting human T, B and NK cells, monocytes or dendritic cells (DCs) (10). Nevertheless, B7-H3 expression could be induced in DCs, monocytes, T, B and NK cells *in vitro* (10, 11). Human B7-H3 is a type-1 transmembrane protein with a short cytoplasmic tail and several unknown signaling motifs (10). In addition to the single extracellular IgV- and IgC-like domains typical of B7 family members, a dual IgV-IgC structure was found in human B7-H3, designated as 4Ig-B7-H3 (11) or B7-H3b (12). Mouse B7-H3 (mB7-H3) was cloned recently (12,

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13), and it had 88% amino acid sequence identity with human B7-H3, but the dual IgV-IgC structure was not found in the mouse genomic DNA (12, 13). The receptor for B7-H3 is unknown thus far, but the counter-receptor was observed to be rapidly induced following T lymphocyte activation in both human and mouse, which was distinct from CD28, CTLA-4, ICOS, and PD-1, because the fusion protein B7-H3-Ig could bind to activated T cells (10, 13). Discrepant results for the function of B7-H3 have been reported.

In current study, we have demonstrated a role for soluble B7-H3 associated with DC allo-stimulatory capability. We found that soluble B7-H3 could inhibit the allo-stimulatory capability of DCs by blocking the secretion of IL-2 and INF-γ in allogenic T cells.

Materials and Methods

Animals and cell lines

BALB/c (H-2^d) mice were obtained from Institute of Organ Transplantation Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China. Adult male mice of 8-12 weeks were used for the study. The DC2.4 dendritic cell line (H-2^b, kindly presented by Dr. Cong-Yi Wang from the Georgia's Health Sciences University, USA) originated from C57BL/6 mice was developed by superinfecting GM-CSF-transduced bone marrow cells with *myc* and *raf* oncogenes as previously described (14). DC2.4 cells express B7-1 and B7-2 and display a dendritic morphology and, as a result, they can be used to present antigen on both MHC class I and class II molecules. Normal phagocytosis function of DC2.4 was shown by their capability to rapidly internalize latex beads (14).

R*eagents*

Goat anti-mouse B7-H3 polyclonal antibody was purchased from R&D system (USA). PE labelled anti-mouse CD83 monoclonal antibody was obtained from eBioscience (USA). PE labeled anti-mouse CD80, streptavidin labeled anti-mouse CD86 monoclonal antibodies and FITC labeled biotin were purchased from BD Biosciences (USA). Polyclonal goat anti-human IgG and HRP labeled anti-human IgG1 monoclonal antibodies were obtained from Zhongshan Biotechnology Co. Ltd. (Beijing, China). Standard human IgG was obtained from Dade Behring Marburg GmbH (Germany). Restriction enzymes, T4 DNA ligase, *Taq* DNA polymerase and pGEM-T easy vector were purchased from Promega (USA). DNA and protein markers were obtained from TaKaRa Biotech (Dalian, China). Gel extraction mini kit, plasmid mini kit and Lipofectamine^{TM}2000 were purchased from Invitrogen (Shanghai, China). CFSE was obtained from Molecular Probes (Portland, OR). BM Chemiluminescence Western blotting kit (Mouse/Rabbit) was obtained from Boehringer Mannheim (Germany). ELISA kits for cytokine detection were purchased from Jingmei Biotech Co. Ltd. (Shenzhen, China).

Establishment of expression construction for mB7h3-hIg

Total RNA from human peripheral blood mononuclear cells (PBMCs) were used for preparation of cDNA with an oligo$(dT)_{18}$ primer according to the manufacturer's instruction. Human IgG1 Fc (hIg) gene was then amplified from cDNA using primers 5'-TTT TCG GAT CCA TGG ACA AAA CTC ACA CAT G-3' and 5'-TTT GGC TCT AGA GCA CTC ATT TAC CCG GGG ACA G-3'. The extracellular domains for mouse B7-H3 (mB7-H3) were amplified from a pcDNA3.1-B7h3 plasmid (kindly presented by Woong-Kyung Suh) (15). The PCR products for mB7-H3 (746 bp) and hIgFc (704 bp) were subcloned into the pGEM-T easy vector with *Hind*III/*Bam*HI and B*am*HI/*Xba*I cutting sites, respectively. The recombinant vectors pGEMT-hIgFc and pGEMT-mB7h3 were confirmed first by restriction enzyme digestion and then by direct DNA sequencing (Invitrogen, Shanghai, China).

For construction of the pmB7h3-hIg expression vector, the DNA fragment for hIgFc was first cleaved from the pGEMT-hIg plasmid by *Bam*HI and *Xba*I digestion and then cloned into the pcDNA3.1(+) vector. DNA fragment for mB7-H3 was obtained by digestion of the pGEMT-mB7h3 plasmid with *Hind*III and *Bam*HI, and then cloned into the upstream region of hIgFc using the same cutting sites within the pcDNA3.1(+) vector. DNA inserts were further confirmed by restriction enzyme digestions and DNA direct sequencing as above.

Transfection, screening and detection of expression of mB7h3-hIg in DC2.4

DC2.4 cells were plated in 24-well tissue culture plate (2×10^5 cells/well) in RPMI 1640 medium supplemented with 10% FCS (Gibco, USA) and 1% penicillin/streptomycin. The pmB7h3-hIg plasmid was transfected into DC2.4 cells by Lipofectamine^{TM}2000 according to the manufacturer's instruction. After 72 h of transfection, the cells were selected with G418 (800 μg/ml, Sigma, USA) for four weeks for establishment of stable transfectants (mB7h3/DC). An empty pcDNA3.1(+) vector was stable transfected into DC2.4 used as a control (C3.1/DC). The expressions of soluble mB7-H3 transient and stable cells were confirmed by ELISA and Western blot analyses using the cultural supernatants. The relative amount of soluble mB7-H3 was determined by ELISA with the human IgG standard preparations. mRNA for recombinant mB7-H3 expression was analyzed by RT-PCR using primers originated from mB7-H3, 5'-CGC TCT TCC CTG ACC TGT T-3' and 5'-CTC CCT GTC CAT CCT TCC A-3'. PCR amplification would produce a DNA fragment of 270 bp.

Determination of cell cycle and apoptosis and the expression of co-stimulatory molecules of DC2.4 cells

Cell cycle and apoptosis of DC2.4 were analyzed by flow cytometry using PI staining. Surface co-stimulatory molecules (CD80, CD86 and CD83) expression on DC2.4 was analyzed by flow cytometry, too. Briefly, DC2.4 cells stably trasfected or no trasfected were plated in 24-well tissue culture plate (2×10^5 cells/well), respectively, in RPMI 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin and cultured at 37°C in 5% CO_2 for

24 h. Subsequently, the cells were harvested and washed twice with 0.1 M PBS (pH 7.4). Some of the cells were stained with PI for the analysis of cell cycle and apoptosis, and others were stained with antibodies conjugated with fluorescence for the analysis of the expression of co-stimulatory molecules.

Splenic T cell purification and CFSE labeling

Splenic lymphocytes were isolated from spleens of BALB/c (H-2^d) mice by gradient centrifugation with Ficoll solution (d = 1.088). The cells were incubated at 37°C in 5% CO_2 in plastic tissue culture flasks for 45 min, nonadherent cells were then harvested and suspended in RPMI 1640 (Sigma, USA) supplemented with 10% FCS. Subsequently, the cells were loaded onto a nylon wool column, and incubated for 45 min. T cells were eluted by cold RPMI 1640 medium. The purity of $CD3^+$ T cells isolated by this approach ranged from 85% to 94% as determined by flow cytometry. T cells were then labeled with CFSE as previously described (16). Briefly, T cells were incubated with CFSE at a final concentration of 5 μM in PBS at room temperature for 10 min followed by centrifugation. The cells were further washed three times with PBS and then re-suspended in RPMI 1640 media contain 10% FCS.

Mixed leukocyte culture (MLC)

CFSE labeled splenic T cells from BALB/C mice were plated in 96-well plates (2×10^5 cells/well) in RPMI 1640 medium supplemented with 10% FCS. DC2.4 stable tranfectants expressing soluble mB7-H3 (4×10^3 cells/well) were added into the cultures in triplicates. Addition of DC2.4 stable transfectants with a pcDNA3.1 vector and no transfected DC2.4 were used as controls. After 3- to 5-day of culture, nonadherent cells were harvested for allogenic T cell proliferation assays by flow cytometric analysis.

Measurement of cytokines

Supernatants from the fifth day of MLC were subject to ELISA analysis for determination of the production of IL-2 and IFN-γ by allogenic T cells using a kit as instructed (Genzyme, USA).

Statistical analysis

For pairwise comparisons, the groups were analyzed using a Student's *t* test, and for multiple comparisons, a one-way ANOVA with Dunnett's method was used. In both cases, $p < 0.05$ was considered significant.

Results

Cloning and construction of expression vector of soluble mB7h3-hIg

PCR amplification using cDNA derived from human PBMCs total RNA yielded a 704 bp DNA fragment corresponding to the expected size for the IgG1 Fc gene (Figure 1A). The fragment was cloned into the pcDNA3.1 vector using the *Bam*HI and *Xba*I cutting sites. A 746 bp DNA fragment corresponding to the extracellular region of the mB7-H3 gene was obtained by PCR amplification of the pcDNA3.1-B7h3 plasmid and was cloned into the upstream region of the hIgFc gene in pcDNA3.1 vector using the *Bam*HI and *Hind*III cutting sites. All of the inserts were confirmed by direct DNA sequencing (Invitrogen. Shanghai, China). After *Hind*III and *Bam*HI, *Hind*III and *Xba*I, *Bam*HI and *Xba*I digestions, the recombinant eukaryotic expression vector pmB7h3-hIg released DNA fragments corresponding to the expected sizes (Figure 1B), indicating that the plasmid was correctly constructed.

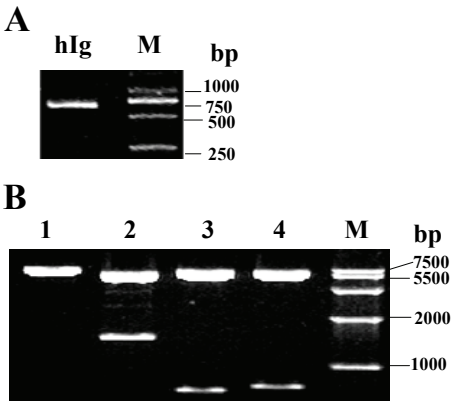

Figure 1. Amplification of human IgG1 Fc gene by RT-PCR and construction of pmB7h3-hIg recombinant vector. (A) Amplification of human IgG1 Fc gene from human PBMCs by RT-PCR. hIg, human IgG1 Fc gene (704 bp); M, DNA marker. (B) Construction of pmB7h3-hIg recombinant vector. Lane 1, pmB7h3-hIg was digested by *Hind*III only; Lane 2, pmB7h3-hIg was digested by *Hind*III and *Xba*I, releasing a 1,450 bp fragment of mB7h3-hIg; Lane 3, pmB7h3-hIg was digested by *Bam*HI and *Xba*I, releasing a 704 bp fragment of hIgFc; Lane 4, pmB7h3-hIg was digested by *Hind*III and *Bam*HI, releasing a 746 bp fragment of mB7-H3 extracellular region.

Expression of mB7h3-hIg fusion protein in DC2.4

DC2.4 stable transfectants for pmB7h3-hIg secreted a soluble fusion protein (mB7h3-hIg) into cultural medium. A 270 bp fragment was amplified from the total RNA of B7h3/DC by RT-PCR corresponding to anticipated size. However, RT-PCR failed to amplify the target sequence using cDNAs derived from no transfected DC2.4 (nDC) and empty pcDNA3.1(+) vector stable transfected DC2.4 (C3.1/DC) (Figure 2A). Western blot analysis of cultural medium using a polyclonal antibody specific for mB7-H3 detected a 49.7 kD protein, which is in agreement with the molecular weight of addition mB7-H3 and hIgFc (Figure 2B). The amount of soluble B7-H3 from 6-day cultures of 1×10^4 B7h3/DC cells was determined by ELISA and ranged from 15 μg/L to 200 μg/L (Figure 2C).

Effect of soluble mB7h3-hIg on the cell cycle and apoptosis and the expression of CD80 and CD86 of DC2.4

To determine the character of DC2.4, its morphous was

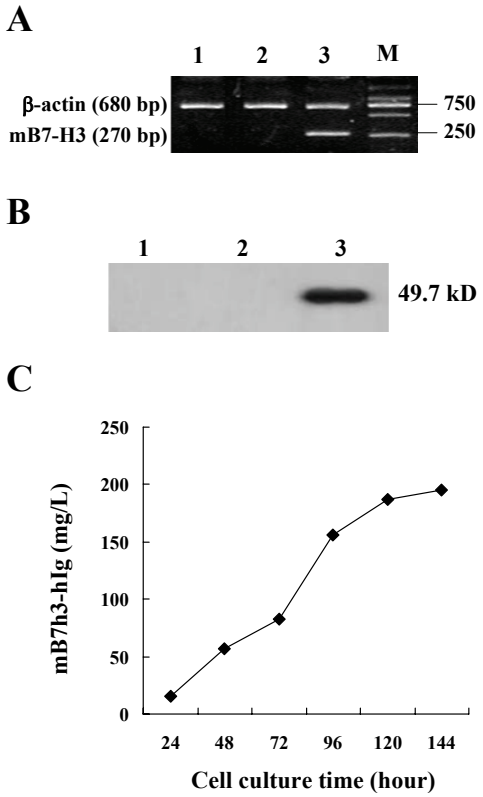

Figure 2. Identification of mB7h3-hIg mRNA and fusion protein expression. (A) Analysis of the expression of mB7-H3 mRNA in DCs by RT-PCR. Lane 1, nDC; Lane 2, C3.1/DC; Lane 3, mB7h3/DC. (B) Detection of mB7h3-hIg fusion protein in cell culture supernatant by Western blot. Lane 1, nDC; Lane 2, C3.1/DC; Lane 3, mB7h3/DC. (C) Determination of mB7h3-hIg in cell culture supernatant of 1×10^4 mB7h3/DC cells cultured for 144 h by ELISA.

observed under light microscopy and the expressions of co-stimulatory molecules CD80, CD86 and CD83 on it were determined by flow cytometery. DC2.4 cells display a typical dendritic profile and express high levels of CD83, CD80 and CD86 (data not shown), indicating a matured DC phenotype for DC2.4 cells.

Cell cycle and apoptosis were analyzed by flow cytometry using PI staining for investigation of the effect of soluble mB7-H3 on cell growth. To determine whether soluble mB7h3-hIg affects the expression of co-stimulatory molecules, the expression of CD80 and CD86 on DCs were tested by flow cytometry, too. We failed to detect the effect of soluble mB7h3-hIg on cell cycling and apoptosis (data not shown) and the expression of CD80 and CD86 of DC2.4 (Figure 3).

Effect of soluble mB7-H3 on allogenic T cell proliferation and cytokine production

We used splenic T cells derived from BALB/c mice as responders for the MLC system. The cells were harvested after 3- or 5-day culture for T cell proliferation assays and detection of cytokine production by allogenic T cells,

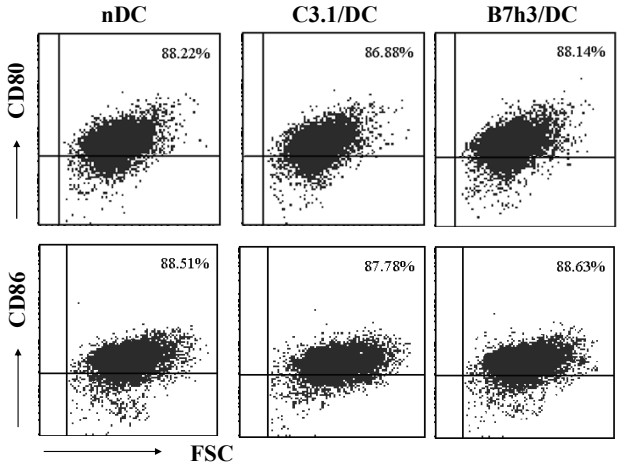

Figure 3. Determination of the expression of CD80 and CD86 on pmB7h3-hIg transfected DCs by flow cytometry. There were no significant differences between B7h3/DC *vs* nDC or C3.1/DC.

respectively. It was found that DC2.4 cells possessed high capability for activation of allogeneic T cells. For 3-day MLC, although soluble mB7h3-hIg showed a minor inhibitory effect on allogenic T cell proliferation and cytokine production, as shown in Figure 4, T cells cultured with DC2.4 cells expressing soluble mB7h3-hIg showed lower proliferation and lower IL-2 and IFN-γ production, and the results were still statistically significant ($p < 0.05$). To our surprise, soluble mB7h3-hIg significantly inhibited allogenic T cell response in 5-day MLC. As shown in Figure 5, T cells co-cultured with DC2.4 cells expressing soluble mB7h3-hIg have significantly lower proliferation and IL-2 and IFN-γ production ($p < 0.01$).

Discussion

Mature DCs, as other professional APC, express co-stimulatory molecules CD80, CD86 and MHC II molecule highly (1-3). CD28, expressed on naïve T cell, is a receptor of CD80 and CD86. B7-CD28 interaction plays key roles in the activation of immune responses upon TCR binding of a peptide-MHC complex (1-3). CTLA-4, a second B7 receptor on T cells with homology to CD28, competes with CD28 for binding to CD80 and CD86. Blockade of B7/CD28 interactions with CTLA-4-Ig is effective in preventing disease in various models of immune-mediated injury, including transplant rejection (17, 18). However, although the importance of the pathway in primary T cell activation is well established, it appears less significant to the generation and maintenance of memory and effector T cell functions (19), and B7-dependent but CD28- and CTLA-4-independent forms of co-stimulation may occur (20, 21).

B7-H3, one of the B7 family members, was identified recently. The expression of B7-H3 in many nonlymphoid tissues indicates that B7-H3 may play an important role in the maintenance of self-tolerance. However, the exact

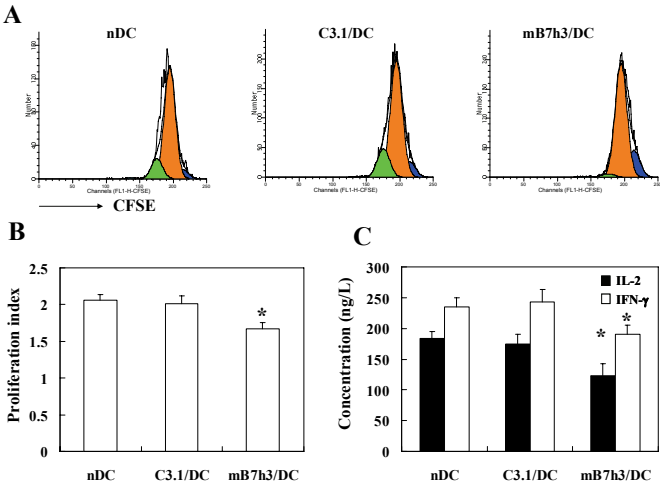

Figure 4. mB7h3-hIg down-regulated co-stimulatory action of DCs to the proliferation and production of IL-2 and IFN-γ of allogenic T cells in MLC for 3 days. (A) The proliferation of allogenic T cell determined by CFSE labeling in MLR for 3 days. Allogenic T cell stimulated by nDC, C3.1/DC or mB7h3/DC, respectively. (B) T cell proliferation index in each group. (C) The production of IL-2 and IFN-γ in cell culture supernatant in every group determined by ELISA. T cell proliferation index and the production of IL-2 and IFN-γ stimulated with B7h3/DC were lower than that stimulated with nDC and C3.1/DC. All the data represent mean ± SD from three independent experiments of nine samples. *, $p < 0.05$ between B7h3/DC *vs* nDC or p3.1/DC and

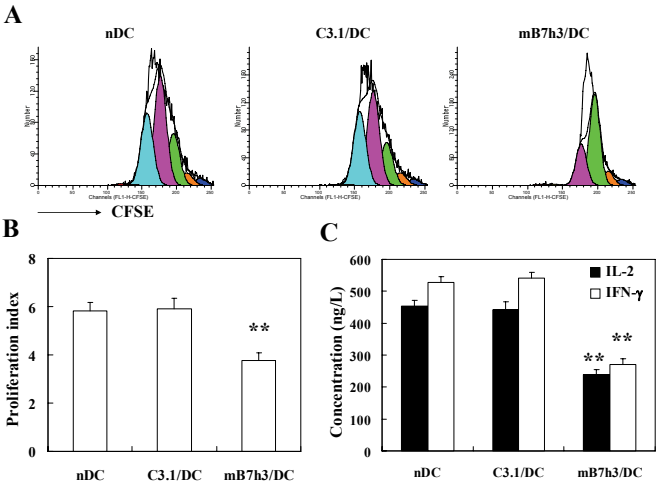

Figure 5. mB7h3-hIg down-regulated co-stimulatory action of DCs to the proliferation and production of IL-2 and IFN-γ of allogenic T cells in MLC for 5 days. (A) The proliferation of allogenic T cell determined by CFSE labeling in MLR for 3 days. Allogenic T cell stimulated by nDC, C3.1/DC or mB7h3/DC, respectively. (B) T cell proliferation index in each group. (C) The production of IL-2 and IFN-γ in cell culture supernatant in every group determined by ELISA. T cell proliferation index and the production of IL-2 and IFN-γ stimulated with B7h3/DC were lower than that stimulated with nDC and C3.1/DC. All the data represent mean ± SD from three independent experiments of nine samples. **, $p < 0.01$ between B7h3/DC *vs* nDC or p3.1/DC.

function of B7-H3 has to be established yet. Discrepant results have been reported. Chapoval et al. demonstrated that soluble hB7-H3 enhanced proliferation of $CD4^+$ and $CD8^+$ T cells, and selectively stimulated IFN-γ mRNA expression in the presence of TCR signaling (10). Soluble mB7-h3 was also found to bind to activated but not resting mouse T cells (12). More recently, Wang et al. reported the participation of B7-H3 in transplantation rejection and suggested that B7-H3 might promote acute and chronic allograft rejection (22). On the contrary, Suh et al. found that B7-H3 might have a suppressive effect on mouse T cell activation and function (15). Their results indicated that mB7-H3 could be involved in the feedback inhibition of Th1-mediated immune responses. Similarly, Prasad et al. reported a negative regulatory effect of B7-H3 on T cells (23). Interestingly, Zhang et al. found that bacteria expressed human recombinant B7-H3 only had a minor enhancing effect on T cell proliferation but strong effect on IL-10 secretion, indicating that B7-H3 could be an important target for regulation of immune response (24).

In this study, The DC cell line DC2.4, expressed co-stimulatory molecules CD80, CD86 and CD83 highly, and could stimulate allogenic T cell proliferation and promote IL-2 and IFN-γ production, suggesting that this DC cell line was a strain of mature DCs and had allogenic stimulatory capability to T cells (14). We had constructed a eukaryotic expression vector (pmB7h3-hIg) for soluble mB7h3-hIg expression. The plasmid could be stably transfected into DC cell line, DC2.4 cells. Western blot and ELISA analyses demonstrated that the transfected cells released high levels of soluble mB7h3-hIg into the cultural medium. These DCs had lower co-stimulatory capability to allogenic T cells proliferation and production of IL-2 and IFN-γ than no transfected DC2.4 and pcDNA3.1(+) transfected DC2.4. It was also found that COS-7 cell-derived soluble mB7h3-hIg could inhibit T cell proliferation stimulated by ConA, but human IgG as control had no effect (data unpublished). Flow cytometric analysis of cell cycle and apoptosis in tranfected and no transfected DCs revealed that transfection of pmB7h3-hIg gene had no effect on the cell cycle and apoptosis of DC2.4. These results indicated that the lower stimulatory capability of pmB7h3-hIg transfected DCs to allogenic T cell proliferation and production of IL-2 and IFN-γ was mediated by soluble mB7h3-hIg, but not by hIg Fc. The receptor of B7-H3 was mainly expressed on activated T cells (10). In the double MLC system, T cells were activated by DCs and expressed B7-H3 receptor. Meanwhile pmB7h3-hIg transfected DCs released mB7h3-hIg fusion protein, and then the soluble fusion protein bonded to its receptor expressed on the surface of activated T cell, and mediate inhibitory signal to inhibit T cell proliferation further. So in this study, the proliferation action of allogenic T cells stimulated by pmB7h3-hIg transfected DC2.4 was decreased more significantly in late phase (for 5 days) ($p < 0.01$), but less significantly in early phase (for 3 days) ($p < 0.05$). More

recently, Zhang et al. found constitutive expression of human B7-H3 at a high level on immature DCs and mature DCs derived from monocytes (25), but we failed to detect the expression of mouse B7-H3 mRNA in the DC cell line by RT-PCR, indicating that DC2.4 did not express B7-h3, which might be contributed to the reason that allogenic T cells had higher proliferation and production of IL-2 and INF-γ stimulated with pmB7h3-hIg transfected DCs in early stage than in later stage. Furthermore, in the double MLC system, the incipient rate of DC was lower, leading to lower concentration of soluble mB7h3-hIg at early phase, which reflected the less early inhibitory action to allogenic T cells activation. Very interestingly, it was also found that the concentrations of IL-2 and INF-γ in MLC supernatant of allogenic T cell cultured with pmB7h3-hIg transfected DCs were obviously lower than those with no gene transfected DCs and pcDNA3.1(+) transfected DC in this study. IL-2 and INF-γ are produced by T cells mainly and they are among the most important cytokines to promote T cell activation and proliferation. For this reason, the results of this study suggested that soluble mB7h3-hIg inhibited allogenic T cell proliferation through IL-2 or INF-γ signal pathway.

In conclusion, we obtained a stable DC strain expressing soluble mB7h3-hIg, successfully. This DC strain could secrete soluble mB7h3-hIg and may be a useful tool for the future studies on the function of mouse B7-H3. We also reported that soluble mB7h3-hIg could down-regulate allogenic T cells proliferation and production of IL-2 and INF-γ. The down-regulatory effect of soluble B7-H3 on allogenic T cells proliferation might be effective *via* blocking the secretion of IL-2 and INF-γ in allogenic T cells. Our characterization of the inhibitory effect of soluble mB7h3-hIg on T cell activation may have great potential for developing a novel strategy for prevention graft rejection in organ transplantation.

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