A Co-inhibitory Molecule, B7-H4, Synergistically Potentiates Oral Tolerance by Inducing CD4+CD25+FoxP3+ T Cells

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Background: A co-inhibitory molecule, B7-H4, is believed to negatively regulate T cell immunity by suppressing T cell proliferation and inhibiting cytokine production. However, the mechanism behind B7-H4-mediated tolerance remains unclear. **Methods:** Balb/c (H-2^d) mice were fed with dendritic cell line, DC2.4 (H-2^b) every day for 10 days. Meantime, mice were hydrodynamically injected with recombinant plasmid expressing B7-H4 fusion protein (B7-H4.hFc) or hFc via tail vein. One day after last feeding, mice were immunized with allogeneic B6 spleen cells. 14 days following immunization, mice were challenged with B6 spleen cells to ear back and the ear swelling was determined the next day. Subsequently, a mixed lymphocyte reaction (MLR) was also performed and cytokines profiles from the reaction were examined by sandwich ELISA. Frequency of immunosuppressive cell population was assayed with flow cytometry and mRNA for *FoxP3* was determined by RT-PCR. **Results:** Tolerant mice given plasmid expressing B7-H4.hFc showed a significant reduction in ear swelling compared to control mice. In addition, T cells from mice given B7-H4.hFc plasmid revealed a significant hyporesponsiveness of T cells against allogeneic spleen cells and showed a significant decrease in Th1 and Th2 cytokines such as IFN-γ, IL-5, and TNF-α. Interestingly, flow cytometric analysis showed that the frequency of CD4+CD25+FoxP3+ Tregs in spleen was increased in tolerant mice given recombinant B7-H4.hFc plasmid compared to control group. **Conclusion:** Our results demonstrate that B7-H4 synergistically potentiates oral tolerance induced by allogeneic cells by increasing the frequency of FoxP3+ CD4+CD25+ Treg and reducing Th1 and Th2 cytokine production. **[Immune Network 2008;8(1):21-28]**

INTRODUCTION

Co-signaling molecules, comprising immunoglobulin and tumor-necrosis factor (TNF) superfamily, play key roles in T cell immunity and tolerance (1). B7:CD28 family are well known to finely regulate T cell responses by delivering co-stimulatory or co-inhibitory signals into T cells, leading to effective T cell activation, differentiation, and prevention of anergy, or down-regulation of T cell immunity, respectively (2). Among the family, B7.1 (CD80) and B7.2 (CD86) are well known co-signaling ligands that are expressed particularly on antigen presenting cells (APCs), whose functions are to prime, activate, and sustain T cell response by delivering positive co-signals into T cells through CD28 (3). The molecules also attenuate, suppress, and terminate T cell response via cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) on T cells, inducing tolerance and preventing autoimmunity (4). Recently, in vivo and in vitro studies showed that soluble CTLA-4 Ig triggers IFN-γ production and subsequently induces tryptophan-catabolizing enzymes such as indoleamine 2,3-dioxygenase (IDO) and tryptophanyl-trna-synthetase (TTS) in B7-expressing DCs (5,6). Therefore, CTLA-4-mediated reverse signaling through B7 molecules renders B7-expressing DCs tolerogenic, leading to allogeneic islet transplant tolerance (5). Thus, bidirectional signals from B7:CD28 pathway, complicating the regulation of immune network, finely tunes the immune response in vivo.

A newly identified B7-H4 plays a negative role in T cell immunity by suppressing T cell expansion and inhibiting Th1

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This work was supported in part by the 1997 Inje University Research Grant by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (No. R13-2007-023-00000-0).

Keywords: B7-H4, oral tolerance, delayed type hypersensitivity

and Th2 cytokines including IFN-γ, IL-2, and IL-4. For example, treatment of GVHD-induced mice with B7-H4 fusion protein (B7-H4.hFc) results in attenuated cytotoxic activity of cytotoxic T lymphocytes (CTLs) against allogeneic antigens. Unexpectedly, recent study using B7-H4-deficient mice indicates that B7-H4 plays a minimal immunoregulatory role in vivo as demonstrated by the mild augmentation of Th1 responses and slight decrease of parasite burdens upon Leishmania infection in B7-H4 knockout mice compared to the wild type mice (7). However, human ex vivo studies reveal that tumor macrophage-associated B7-H4 that was upregulated by IL-10 in tumor microenvironment greatly suppresses antigen-specific T cell immunity (8,9). There are still many questions to be answered about the physiological role of endogenous B7-H4 in many clinical diseases such as autoimmune diseases, allergy, graft rejection, and cancer.

It has been shown that oral administration of a wide variety of non-pathogenic antigens suppresses subsequent systemic immune responses depending on the nature and dose of the fed antigen (10). A number of transplantation studies using cytokine gene-deficient mice demonstrate that type I cytokine IFN-γ and type 2 cytokine IL-4 are responsible for the development of tolerance as evidenced by the increased delayed-type hypersensitivity (DTH) in IFN-γ and IL-4 knockout mice (11). In addition, Zelenika et al reported that TGF-β plays a protective role in allograft rejection, as shown by the prevention of male skin grafts that was transplanted into female mice with HY-specific TCR transgene (12). In light of the immunoregulatory role of B7-H4 in alloresponse, we investigated whether B7-H4 is involved in the induction of oral tolerance and the possible mechanism by which B7-H4 renders mice more tolerant to alloantigen.

MATERIALS AND METHODS

Mice

6 to 8 week-old female mice of C57BL/6 or BALB/c, purchased from the Jackson Laboratory (Maine, USA), were housed in specific pathogen-free (SPF) conditions at the animal facility of Inje University College of Medicine and used in experiments under approval from the Institutional Animal Care and Use Committee (IACUC).

Hydrodynamic injection of plasmid DNA

Genes encoding murine B7-H4 fused to human Fc (B7-H4.hFc) were cloned into pCMV1 vector (Kodak, USA), in which FLAG tag sequence was attached to N-terminal of fusion protein. This FLAG tag was used to identify the fusion protein by enzyme-linked immunosorbent assay (ELISA). Recombinant plasmid DNAs were prepared using the Endo-Free Plasmid System (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One hundred microgram of plasmid DNA was injected into tail vein of mouse in a volume of phosphate buffered saline (PBS) equivalent to 8% of the mouse body weight. Fusion protein released into the blood was measured by ELISA.

Sandwich ELISA for fusion protein in blood

Blood was serially drawn from the tail vein and heparinized with 20 unit/ml heparin. Plasma was prepared following centrifugation at 13,000 rpm for 4 minutes and stored at -80°C until use. The level of soluble B7-H4 was measured in accordance to a general ELISA protocol. Briefly, ELISA plates were coated with anti-flag antibody (2 μg/ml) in 0.1 M carbonate buffer at 4°C overnight. Plates were then blocked with 1% bovine serum albumin (BSA) in 0.05% Tween-20/PBS for 1 hr at room temperature. Serially diluted plasma samples were added into each well and incubated at room temperature for 2 hr. After washing with PBS, each well was incubated with horseradish peroxidase-conjugated anti-human IgG at room temperature for 1 hr. Color reaction was developed with 3, 3', 5, 5'-Tetramethyl-benzidine (TMB) Liquid Substrate (Sigma, USA), and then stopped with 0.1 M H_2SO_4. The absorbance at 450 nm was measured using an ELISA plate reader (MicroBeta® TriLux, PerkinElmer).

Cell culture

The DC2.4 cell line was kindly provided by Dr. Kenneth L. Rock, University of Massachusetts. The cell line was grown in RPMI 1640 medium, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% FBS (Invitrogen).

Flow cytometry

DC2.4 cells suspended in FACS buffer (PBS containing 1% BSA and 20 mM sodium azide) were incubated with monoclonal antibody (mAb) specific for Fc receptor (clone 2.4G2) to block Fc receptor on the cell surface at 4°C for 20 min. Then, cells were incubated with fluorescein isothiocynate (FITC)-labeled anti-mB7-H4 (clone 8H3) for 20 min at 4°C and washed three times with FACS buffer. Cells were analyzed by flow cytometry using the CellQuest program on a

FACSCalibur (BD Biosciences, USA). In some experiments, CD4+ T cell were purified from splenocytes by using the MACS system (Milteny Biotec GmbH, Bergisch Gladbach, Germay). The purity of purified CD4+ T cells was >90%. To stain the each cell population, cells were stained with FITC-, PE-, or PE-Cy5-conjugated mAb against cell-specific markers such as CD3, CD4, B220, CD11c, CD11b, and CD25 (BD Biosciences), and then analyzed with FACSCalbur (BD Biosciences). For detection of Tregs, cells were stained with PE-Cy5-conjugated anti-CD4, FITC-conjugated anti-CD25, and PE-conjugated anti-FoxP3.

Induction of oral tolerance and delayed type hypersensitivity (DTH) assay

Balb/c mice were fed daily with 2×10^5 DC2.4 cell line for 5, 7, or 10 days using gavage tube following the schedule (Fig. 1). To see the effect of B7-H4 on oral tolerance induction, the mice were injected hydrodynamically with recombinant plasmid encoding B7-H4.hFc three times with 2 day-interval. One day after last feeding, mice were immunized with 5×10^6 splenocytes from C57BL/6 subcutaneously. 2 weeks after immunization, mice were challenged with 1×10^7 B6 splenocytes in 25 μl saline by being injected into right hind side of earback. For control, mice were injected with 25 μl of saline into left hind side of earback. After 24 hr of challenging, the ear thickness was measured using peacock dial thickness gauge (Model G-1A, Ikemoto Scientific Technology, Japan). DTH was expressed as specific earback swelling, which is calculated as follows: {[24-hr measurement of right hind earback - 0-hr measurement of right hind earback] - [24-hr measurement left hind earback- 0-hr measurement left hind earback]}$\times$ 0.01 mm. Student's t test was used to evaluate the statistical significance of the results.

Mixed-lymphocyte reaction (MLR)

Spleen and mesenteric lymph node cells from oral tolerance-induced BALB/c mice were prepared as follows; red blood cells (RBC) were lysed in Tris-NH4Cl solution. The cells were washed in culture medium containing 10% FCS, resuspended at 3×10^6 cells/ml, and used as a responder. Spleen cells from C57BL/6 mice were prepared as described above and treated with PBS containing 0.5 mg/ml mitomycine-C (Sigma, USA) at 37°C for 20 min, and used as a stimulator. For MLR, purified 3×10^5 of responding lymphocytes were mixed in 96 well V-bottom plate with mytomicine-C-treated C57BL/6 splenocytes at different ratios, and incubated at 37°C with 5% CO_2 for 5 days. Proliferation of responding lymphocytes was determined as incorporation of [^{3}H]-thymidine into the cells by adding 0.5 μCi of [^{3}H]-thymidine per well during the last 16 hour of culture. Incorporated [^{3}H]-thymidine was determined by Packard Topcount Microplate Scintillation counter (Packard Instrument), and expressed as counts per minute (cpm).

ELISA for mouse Th1/Th2 cytokine detection

For detection of cytokines in culture supernatant, conventional sandwich ELISA was performed according to a general procedure. Briefly, 96 well immunoplate (Nunc, Denmark) was coated with capture antibodies including anti-IFN-γ, -TNF-α, and -IL-5 at 4°C overnight. Following blocking each well with 5% FBS in PBS for 1 hr at room temperature, the supernatant was incubated for 2 hr at room temperature. Subsequently the assay was detected by biotin-conjugated detecting antibodies and developed by HRP-conjugated streptavidin and TMB as a substrate. Optical densities were measured at 450 nm with an ELISA plate reader.

Isolation of total RNA and RT-PCR

Total RNA was extracted from spleen cells or CD4+ T cells using TRIzol Reagent (Life Technologies, Frederick, MD), and digested with RNase-free DNase (RQ1 DNase; Promega, Madison, WI) to remove a contaminating genomic DNA. The

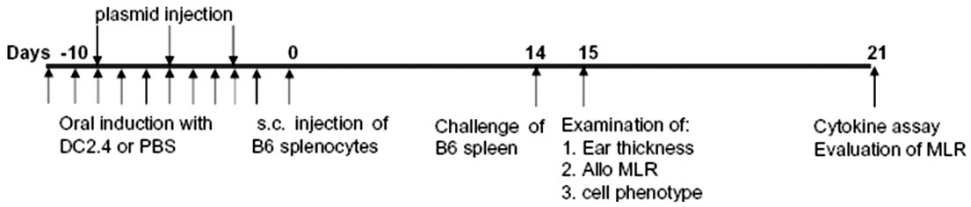

Figure 1. Schedule for induction of oral tolerance, hydrodynamic injection of plasmids, challenge of allogeneic cells, and assays including delayed type hypersensitivity, MLR, and cytokine assay.

first-strand cDNA was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). FoxP3 and glyceraldehydes 3-phophate dehydrogenase (GAPDH) were amplified as follows: 30 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec, and a final synthesis of 7 min at 72°C using target specific primers of FoxP3 sense primer, 5'-CAG CTG CCT ACA GTG CCC CTA G-3', and FoxP3 antisense primer, 5'-CAT TTG CCA GCA GTG GGT AG-3'; GAPDH sense primer, 5'-TTC ACC ACC ATG GAG AAG GC-3', and GAPDH antisense primer, 5'-GGC ATG GAC TGT GGT CAT GA-3'.

Statistics

Statistical significance of difference between control and experimental groups was calculated using the Student's *t*-test.

RESULTS

In vivo expression of B7-H4 fusion protein

We validated the recombinant plasmids expressing B7-H4 fusion protein or its control counterpart, hFc, by transfecting HEK293 cell line and determining soluble fusion protein in the culture supernatant with sandwich ELISA as described in the Materials and Methods (data not shown). B7-H4 fusion protein was also expressed in vivo as demonstrated by the sandwich ELISA using blood samples drawn from the mice hydrodynamically injected with recombinant plasmids via tail vein. In vivo expression of fusion protein was around 200 μg/ml at day 1 postinjection, and then reduced thereafter. They were not detectable after day 5 postinjection (Fig. 2A, B).

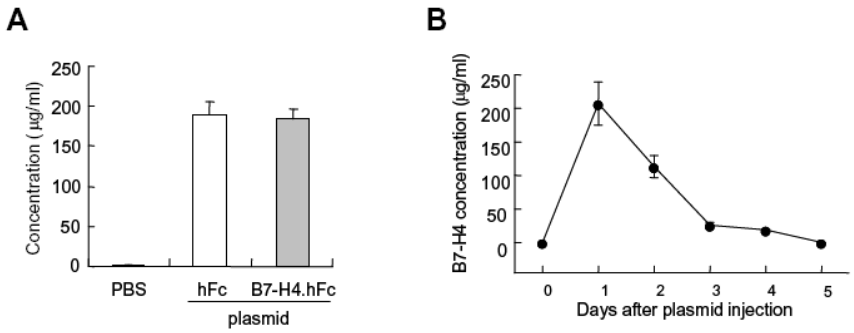

Figure 2. Expression kinetics of recombinant B7-H4.hFc and hFc in blood following hydrodynamic injection of plasmids. The soluble B7-H4.hFc or hFc was measured in accordance to a general ELISA protocol. (A) In vivo expression of recombinant proteins in blood 24 hr after hydrodynamic injection of plasmids. (B) Kinetics of in vivo expression of B7-H4.hFc. Data are representative of three independent experiments.

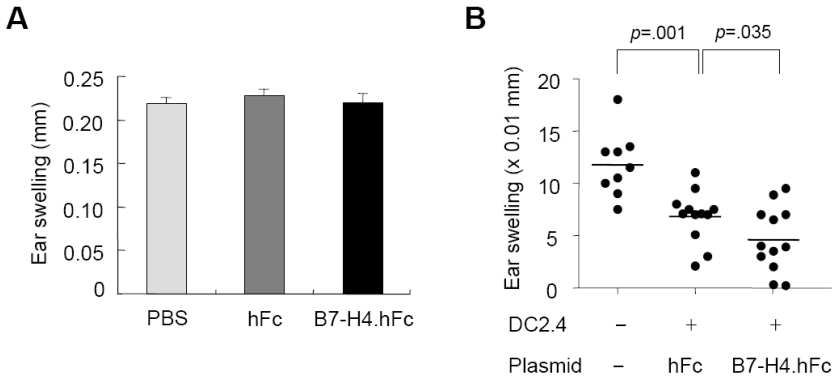

Figure 3. Delayed type hypersensitivity in oral tolerance-induced mice given recombinant plasmids. (A) Measurement of earback swelling before eliciting, and (B) after elicitation in group of tolerant mice given indicated plasmids. Results were expressed as specific earback swelling. All data represent the mean ± SD of three individual animals in each group and are representative of four independent experiments.

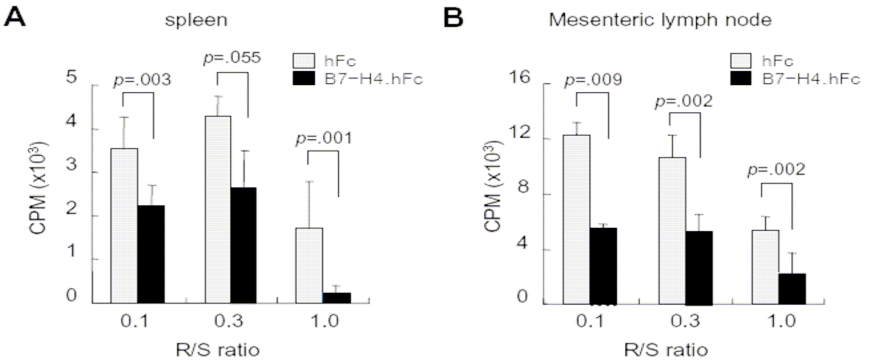

Figure 4. Mixed lymphocyte reaction (MLR) of T cells from oral tolerance-induced mice given recombinant plasmids. Spleen cells (A) and mesenteric cells (B) from tolerant mice (Balb/c) were used as a responder (R) T cells, and mitomycin-treated B6 spleen cells as a stimulator. Results are mean ± SD of triplicate cultures from one of three independent experiments with similar results.

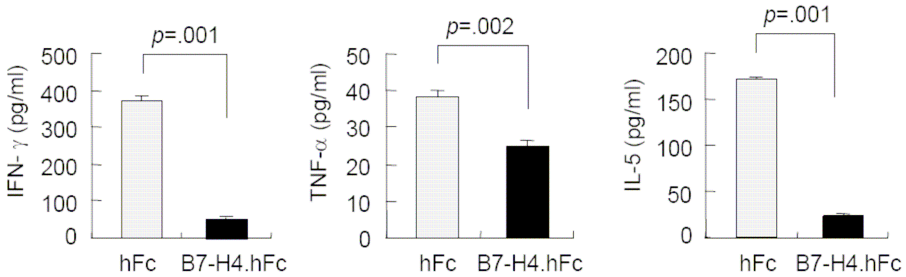

Figure 5. Cytokine profiles of supernatants from mixed lymphocyte reaction. Supernatants from MLR described in Figure 4 were assayed for cytokine profiles with sandwich ELISA. All data represent the mean ± SD of individual animal in each group and are representative of three independent experiments.

B7-H4 synergistically suppressed a DTH response in tolerant mice

Oral tolerance-induced mice given recombinant B7-H4.hFc plasmid showed a significant reduction of DTH response, as demonstrated by the decreased ear swelling compared to those with control plasmid or PBS (Fig. 3A, B). In line with the data, alloresponse of spleen T cells from tolerant mice injected with recombinant B7-H4.hFc plasmid was significantly diminished (Fig. 4A). Similar results were observed in the assay using mesenteric lymph node T cells (Fig. 4B).

B7-H4 synergistically potentiates tolerance by inhibiting the production of TNF-α

Having demonstrated that B7-H4 rendered mice more tolerant to allogeneic antigen, we examined the mechanisms behind the synergistic effect of B7-H4 in tolerance induction. We found that Th1 cytokines such as of IFN-γ, TNF-α and Th2 cytokine including IL-5, not IL-4 were decreased in MLR using spleen cells

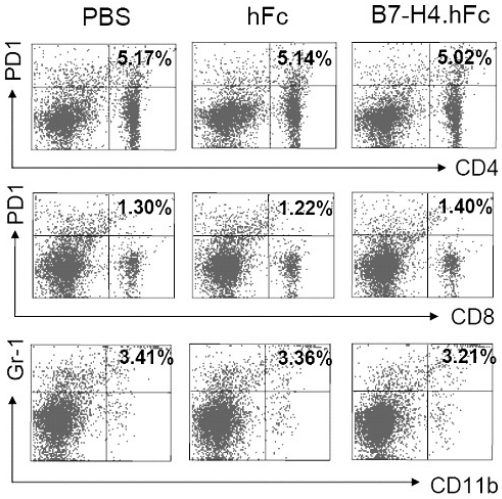

Figure 6. Flow cytometric analysis of immune cell populations. PD-1-expressing CD4+ or CD8+ T cells, and myeloid dendritic cells from tolerant mice given recombinant plasmids were analyzed by flow cytometry. Data are representative of three independent experiments.

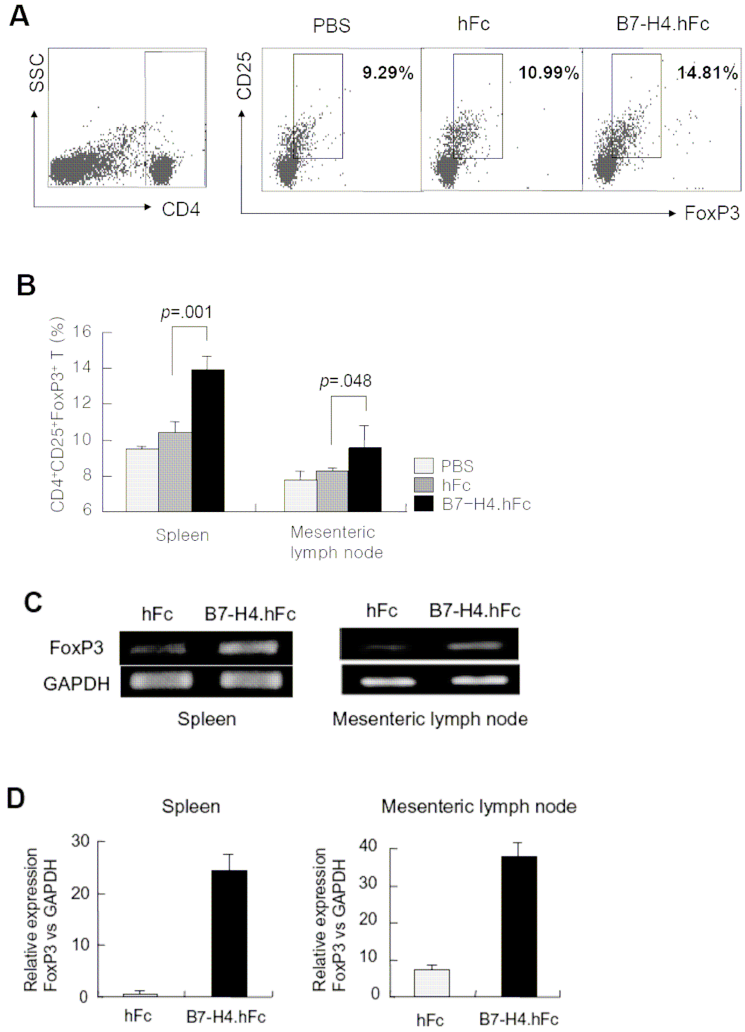

Figure 7. Determination of CD4+CD25+FoxP3+ T cell population. Frequency of CD4+CD25+ FoxP3+ T cells in spleens or mesenteric lymph nodes from tolerant mice given recombinant plasmids were analyzed with flow cytometry (A) and represented as bar graphs (B). Data are representatives of three independent experiments. (C) Expression of FoxP3 was determined by RT-PCR, and represented as bar graphs (D). All data represent the mean ± SD of individual animal in each group and are representative of three independent experiments.

from mice given recombinant B7-H4.hFc plasmid compared to control group (Fig. 5). Specifically, IFN-γ production was remarkably reduced in tolerant mice received recombinant B7-H4.hFc plasmid (53 ± 6 pg/ml) compared to control mice (372 ± 17 pg/ml). However, there was no significant decrease in IL-2 and IL-4 production between the groups.

B7-H4 increases the frequency of CD4+CD25+ FoxP3+ T cells in tolerant mice

Next, we examined the cell population that was responsible for B7-H4-mediated potentiation of tolerance. There was no significant difference in PD-1 expressing T cell population that is known to be increased in a certain condition of immune suppression such as chronic viral infection and cancers (13,14). Furthermore, we found that suppressive cell populations including myeloid suppressive cell population (CD11b+Gr1+) were not also increased in tolerant mice given recombinant plasmid for B7-H4.hFc plasmid compared to those given control plasmid (Fig. 6). Although oral feeding of allogeneic cells augmented the frequency of Treg (CD4+CD25+ FoxP3+) in vivo, B7-H4 greatly increased the frequency of Treg in spleen and mesenteric lymph node from tolerant mice (Fig. 7A, B), which was further confirmed by RT-PCR of *FoxP3* (Fig. 7C, D).

DISCUSSION

In oral tolerance model, it is the dose of antigen fed that de-

termines the mechanisms favoring the peripheral tolerance. It is well known that oral exposure of low doses of antigen generates regulatory T cells that secretes TGF-β, IL-4, and IL-10, whereas high doses of antigen causes anergy or deletion of antigen-specific T cells (15-17).

In this study, oral exposure of mice to allogeneic DC2.4 cell line following hydrodynamic injection of plasmid expressing B7-H4.hFc results in a potentiation of oral tolerance as demonstrated by significantly decreased DTH response and MLR compared to control group given recombinant hFc plasmid. Interestingly, our result indicates that the degree of hyporesponsiveness of tolerant T cells to alloantigen did not depend on total amount of antigen fed since there was no significant difference in DTH and MLR between the groups that were fed with different doses of allogeneic cells (data not shown). Furthermore, the data showed that soluble B7-H4 significantly reduced Th1 cytokines including IFN-γ and TNF-α and Th2 cytokine such as IL-5 in tolerant mice injected with recombinant plasmid for soluble B7-H4.hFc. In contrast to the previous reports indicating that IFN-γ is crucial cytokines to induce tolerance and prevent allograft rejection (18,19), it appeared that mice with an exaggerated tolerance by soluble B7-H4.hFc produced a significantly lower level of IFN-γ than control mice did. The conflicting data might come from a different experiment settings and suggest that there might be other mechanisms responsible for a potentiated tolerance by B7-H4.

Regarding the cell populations that are responsible for the induction and maintenance of oral tolerance, it has been suggested that cells with suppressive function such as CD11c+ CD11b+ DCs (20), NKT cells (21) and CD4+CD25+ Tregs are involved (22). However, in our experiment setting, the frequency of Tregs, not myeloid suppressor cells (MSC), is increased in tolerant mice given recombinant B7-H4.hFc plasmid, suggesting that B7-H4 synergistically potentiates oral tolerance through induction of Treg in vivo. However, how B7-H4 induces Treg in vivo remains unclear.

We employed hydrodynamic injection of plasmid to express soluble B7-H4 Ig in vivo. Besides its convenience in handling, it circumvents a time-consuming effort to prepare fusion proteins with high quality for in vivo use (23,24). In this study, single injection of 100 μg of recombinant plasmid into mice is enough to produce around 200 μg/ml in vivo at 24 hr following injection, which amount is generally adopted by most in vivo experiments using purified target proteins (25). To keep the soluble proteins at certain level in vivo, we performed hydrodynamic injection of the plasmids every third day throughout the feeding period.

Taken together, our results suggest that B7-H4 synergistically potentiates oral tolerance by enhancing Treg population and suppressing Th1 and Th2 cytokines including IFN-γ TNF-α, and IL-5. Therefore, B7-H4 might be of great value for improving the therapeutic efficacy of oral tolerance, and therefore benefit the treatment of allograft rejection in human.

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