Crucial roles of B7-H1 and B7-DC expressed on mesenteric lymph node dendritic cells in the generation of antigen-specific CD4+Foxp3+ regulatory T cells in the establishment of oral tolerance

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Oral tolerance is a key feature of intestinal immunity, generating systemic tolerance to fed antigens. However, the molecular mechanism mediating oral tolerance remains unclear. In this study, we examined the role of the B7 family members of costimulatory molecules in the establishment of oral tolerance. Deficiencies of B7-H1 and B7-DC abrogated the oral tolerance, accompanied by enhanced antigen-specific CD4+ T-cell response and IgG1 production. Mesenteric lymph node (MLN) dendritic cells (DCs) displayed higher levels of B7-H1 and B7-DC than systemic DCs, whereas they showed similar levels of CD80, CD86, and B7-H2. MLN DCs enhanced the antigen-specific generation of CD4+Foxp3+ inducible regulatory T cells (iTregs) from CD4+Foxp3− T cells rather than CD4+ effector T cells (Teff) relative to systemic DCs, owing to the dominant expression of B7-H1 and B7-DC. Furthermore, the antigen-specific conversion of CD4+Foxp3− T cells into CD4+Foxp3+ iTregs occurred in MLNs greater than in peripheral organs during oral tolerance under steady-state conditions, and such conversion required B7-H1 and B7-DC more than other B7 family members, whereas it was severely impaired under inflammatory conditions. In conclusion, our findings suggest that B7-H1 and B7-DC expressed on MLN DCs are essential for establishing oral tolerance through the de novo generation of antigen-specific CD4+Foxp3+ iTregs. (Blood. 2010;116(13):2266-2276)

Introduction

The gastrointestinal tract is constantly exposed to a multitude of foreign materials that may be either harmful or beneficial to the organism.1,2 Consequently, the intestinal immune system has to balance protective immune responses to potentially pathogenic microorganisms with nonresponsiveness to commensal bacteria and food antigens to maintain immune homeostasis in this environment, a phenomenon known as oral tolerance.1-4 Although several mechanisms that induce the suppression of antigen-specific immune responses in oral tolerance have been proposed,1 including recessive tolerance mediated by clonal deletion and anergy as well as dominant tolerance involving active immune suppression by CD4+CD25+Foxp3+ regulatory T cells (Tregs)5,7 that encompass self-reactive thymic-derived naturally occurring Tregs (nTregs) and inducible Tregs (iTregs) generated from antigen-specific naive CD4+CD25−Foxp3+ T cells in peripheral areas under certain environmental conditions, the exact molecular mechanisms mediating cellular characteristics in the intestinal mucosa are not yet fully understood.

The outcome and quality of an immune response is dependent on the multiple signals between antigen-presenting cells (APCs) and antigen-specific T cells, including antigen recognition by the T cell antigen receptor (TCR) interacting with peptide-major histocompatibility complex molecules on APCs as well as the provision of cytokines and membrane-bound costimulatory molecules, especially those of the B7-CD28 family.8,9 The classical B7-CD28 pathway includes 2 ligands, B7-1/CD80 and B7-2/CD86 on the APCs and at least 2 receptors, CD28 and cytotoxic T-lymphocyte antigen 4, on the T cells.8,9 More recently identified B7-homologs, including B7-H1/programmed death-ligand 1 (PD-L1), B7-DC/PD-L2, B7-H2/inducible costimulator ligand (ICOSL)/B7h/B7RP-1, B7-H3, and B7-H4/B7x/B7-S1 are expressed on APCs as well as on cells within nonlymphoid organs.8,10 Although B7-H4 remains an orphan, both B7-H1 and B7-DC interact with PD-1, whereas B7-H2 is known to bind to ICOS, and B7-H3, to Trem-like transcript 2.8,10 Although pathways in the B7-CD28 family provide the stimulatory and inhibitory signals needed for the activation, inhibition and fine-tuning of T-cell responses to defend against microbes and regulate self-tolerance, little is known about their precise role in intestinal immunity.

Dendritic cells (DCs) are essential APCs that initiate primary immune response. DCs consist of heterogeneous subsets, including conventional DCs and plasmacytoid DCs, distinguishable by...
surface and intracellular phenotypic markers, immunologic function, and anatomic distribution. Immature DCs (iDCs) serve as sentinels, recognizing the presence of invading pathogens through various pattern-recognition receptors, and become mature DCs (mDCs) with the up-regulated expression of major histocompatibility complex and costimulatory molecules under inflammatory conditions. Consequently, mDCs move via the afferent lymphatics into the T-cell area of secondary lymphoid tissues, where they prime rare antigen-specific naïve T cells for differentiation into effector T cells (Teff), including T helper type (Th)1 cells, Th2 cells, and Th17 cells, depending on environmental conditions. DCs thereby play a crucial role in the link between innate and adaptive immunity. Conversely, accumulating evidence suggests that systemic iDCs are also crucial for the induction of immunologic tolerance under steady-state conditions, and the mechanisms involved include recessive and dominant tolerance in the periphery, a function of likely importance in self-tolerance as well as immune disorders and transplant rejection. Although much attention has been paid to characterizing DCs in the intestinal microenvironment, how the unique propensity of mucosal DCs would influence the establishment of protective immunity versus immune tolerance in the intestine remains poorly understood.

In this study, we addressed the mechanism responsible for the establishment of oral tolerance using mice deficient in B7 costimulatory molecules (B7−/− mice) and knockin mice expressing antigen-specific TCR and enhanced green fluorescent protein (EGFP) under the control of the Foxp3 gene.

**Methods**

**Mice**

BALB/c mice were purchased from Charles River Breeding Laboratories. Cd80/Cd86−/−C57BL/6 mice were purchased from The Jackson Laboratory. B7h1−/−C57BL/6 mice, B7h2−/−/B7h2−/−C57BL/6 mice, and B7h1−/−B7h2−/−C57BL/6 mice have been generated previously. All C57BL/6 mice were backcrossed to the BALB/c background for 9 generations. Foxp3EGFP/C57BL/6 mice were bred with DO11.10 ovalbumin (OVA) TCR transgenic BALB/c mice for 9 generations to obtain Foxp3EGFP/Do11.10 OVA TCR transgenic BALB/c mice. Foxp3EGFP/Rag2−/−/Do11.10 OVA TCR transgenic BALB/c mice were also generated by crossing Foxp3EGFP/Do11.10 OVA TCR transgenic BALB/c mice with Rag2−/−Do11.10 OVA TCR transgenic BALB/c mice.

All mice were used between 6 and 10 weeks of age and maintained in specific pathogen-free conditions and in accordance with guidelines of the Institutional Animal Care Committee of the RIKEN Institute.

**Induction of oral tolerance and immunization with OVA**

Mice (5 per group) were administered intragastrically with 25 mg of chicken OVA protein (A7642-1VL; Sigma-Aldrich) dissolved in phosphate-buffered saline (PBS). Control mice were given PBS alone. One week after OVA feeding, all mice were immunized subcutaneously with 100 μg of OVA protein emulsified in complete Freund adjuvant (Difco). Alternatively, mice were intraperitoneally injected with rat immunoglobulin G (IgG; Sigma-Aldrich) used as control Ig or anti-CD25 monoclonal antibody (mAb; PC61; each 500 μg/mouse) on days −7, −5, −3, and −1 before immunization to deplete CD25+ cells. In another experiment, mice were fed CpG oligodeoxynucleotide (ODN) 1668 (50 μg/mouse, Hokkaido System Science) on days −7 and −3 before immunization. Two weeks after immunization, Sp, MLNs, and sera were obtained from the mice.

**Measurement of serum OVA-specific Ab titers**

Serum OVA-specific IgG1 was assayed by enzyme-linked immunosorbent assay (ELISA) using a Mouse Anti-OVA-IgG1 ELISA KIT (Sibayagi) according to the manufacturer’s instructions.

**Preparation of T cells and CD11c+ DCs**

Spleen (Sp) and MLNs were digested with collagenase type III (Worthington Biochemical) at 37°C for 20 minutes, and single-cell suspensions were obtained by forcing through a 100-μm cell strainer (BD Biosciences). CD4+ T cells or CD8+ T cells were purified with mouse CD4 T lymphocyte Enrichment Set-DM or mouse CD8 T lymphocyte Enrichment Set-DM (both from BD Biosciences), respectively. In some experiments, CD4+ T cells were sorted into CD25+ T cells and CD25+ T cells by FACSVantage. Alternatively, KJ1-26+ T cells were sorted into Foxp3EGFP−CD25+ T cells and Foxp3EGFP−CD25− T cells by FACSVantage (BD Biosciences). CD11c+ DCs were purified by AutoMACS with mouse CD11c (N418) Microbeads (both from Miltenyi Biotec) with high purity (> 97% of CD11c+ cells).

**Flow cytometry**

Cells were stained with fluorescein-conjugated mAbs to mouse CD4 (RM4-5), CD11c (HL3), CD80 (16-10A1), CD86 (GL1), CD103 (M290), CD11c (HL3), CD80 (16-10A1), CD86 (GL1), CD103 (M290), and CD90 (Thy1.1). Fixation-Permeabilization solution (BD Cytofix/Cytoperm kit; BD Biosciences) was used to permeabilize cells during the final 2 hours. Subsequently, the cells were resuspended in Phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% sodium azide. Cells were stained with antibodies to CD4 and CD8, CD11c, and surface and intracellular phenotypic markers, immunologic function, and anatomic distribution.
TGF-β1 (10 ng/mL; Wako Pure Chemicals) and/or retinoic acid (RA; 10nM; Sigma-Aldrich) for 3 days in 96-well flat-bottomed plates. In some experiments, reconstituent mouse IL-6 (20 ng/mL; Wako Pure Chemicals), Cpg ODN 1668 (0.1μM), control rat Ig or anti–PD-1 mAb (RMP1-14)/2 (each 10 μg/mL) was added to the culture. For T cell differentiation in vitro, Rag2−/−KJ1-26+ CD25+ Foxp3EGFP+ T cells (2 × 106) were cultured with Sp CD11c+ DCs or MLN CD11c+ DCs (2 × 105) in neutral conditions in the presence or absence of reconstituent human TGF-β1 (10 ng/mL), reconstituent mouse IL-6 (20 ng/mL) and/or Cpg ODN 1668 (0.1μM) for 3 days in 96-well flat-bottomed plates. Analysis of the expression of Foxp3EGFP+ or IL-17 among gated CD4+ T cells was performed by flow cytometry as described in “Flow cytometry.”

Adoptive transfer

Mice (5 per group) were intravenously injected with Rag2−/−KJ1-26+ CD25+ Foxp3EGFP+ T cells (5 × 106/mouse), and then intragastically administered with OVA (25 mg/mouse) the next day. Control mice were given PBS alone. Alternatively, mice were fed Cpg ODN 1668 (50 μg/mouse) on day 0 and 6 after adoptive transfer. After 11 days, CD4+ T cells were purified from Sp and MLNs in the recipient mice as described in “Preparation of T cells and DC11c+ DCs.” MLN CD11c+ DCs were labeled with carboxyfluorescein diacetate-succinimidyl ester (CFSE; Molecular Probes) at 37°C for 10 minutes, and washed twice with cold PBS. Subsequently, CFSE-labeled Rag2−/−KJ1-26+ CD25+ T cells (3 × 106/mouse) and CD4+ CD25− T cells (106/mouse) obtained from OVA-fed and nonfed mice as described in “Preparation of T cells and CD11c+ DCs,” were intravenously injected into mice (5 per group) 24 hours before the intraperitoneal injection with OVA protein (100 μg/mouse). Three days after the intraperitoneal injection with OVA protein, CD4+ T cells were purified from Sp in the recipient mice as described in “Preparation of T cells and DC11c+ DCs,” and the gated KJ1-26+ CD4+ T cells was analyzed for CFSE dilution to detect the dividing cells by flow cytometry.

Immunohistochemical analysis

Sp and MLNs kept flat were fixed at 4°C in a fresh solution of 4% paraformaldehyde (Wako Pure Chemicals). The samples were then washed in PBS, incubated overnight at 4°C in a solution of 30% sucrose, embedded in OCT compound (Sakura Finetechnical) and frozen in liquid N2. The tissue segments were sectioned with a cryostat at 8μm. Frozen sections were blocked in TNB buffer (PerkinElmer Life Science) containing 5% normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum. To block endogenous biotin, the sections were further treated with the Streptavidin/Biotin Blocking Kit (Vector Laboratories), and normal rat serum.

Statistical analyses

Data are expressed as the mean plus or minus SD. The statistical significance of the values obtained was evaluated by analysis of variance. A P value of less than .01 was considered significant.

Results

Requirement of B7-H1 and B7-DC for establishing oral tolerance

We first investigated the induction of oral tolerance in mice deficient in CD80/CD86, B7-H1, B7-DC and B7-H2. Oral administration of OVA before systemic immunization with OVA markedly suppressed the antigen-specific response of CD4+ T cells and CD8+ T cells as well as IgG1 production in wild-type (WT) mice (Figure 1A-B and supplemental Figure 1). Although CD80/CD86−/− mice and B7h2−/− mice showed a significantly or slightly lower antigen-specific immune response than WT mice, OVA feeding still inhibited the response (Figure 1C,D,I,J and supplemental Figure 1).

In contrast, systemic immunization with OVA caused a greater antigen-specific immune response in B7h1−/− mice and B7dc−/− mice than WT mice, and OVA feeding had little or no effect on the response (Figure 1E-H and supplemental Figure 1).

Next, we examined the impact of the B7 family on the role of CD4+Foxp3+ Treg in oral tolerance. CD80/CD86−/− mice and B7h2−/− mice, but not B7h1−/− mice or B7dc−/− mice, showed a significant or slight reduction in the frequency and the absolute number of CD4+Foxp3+ Treg in Sp and MLNs compared with WT mice (supplemental Figure 2A-B). On the other hand, OVA feeding before systemic immunization with OVA increased the frequency of CD4+Foxp3+ Treg in Sp and MLNs relative to the systemic immunization alone in WT mice, and similar results were observed in CD80/CD86−/− mice and B7h2−/− mice, but not in B7h1−/− mice or B7dc−/− mice (supplemental Figure 2C-D).

Furthermore, depletion of CD4+CD25+Foxp3+ Treg in Sp and MLNs with anti-CD25 mAb before systemic immunization with OVA markedly enhanced the antigen-specific immune response in OVA-fed and nonfed WT mice (supplemental Figure 3).

Involvement of B7-H1 and B7-DC in the suppressive effect of MLN DCs

Accumulating evidence supports the concept that MLNs are privileged sites in triggering oral tolerance, where DCs possibly migrate from lamina propria (LP) after sampling antigens present these antigens to naive CD4+ T cells. Although mucosal DCs are suggested to be immune suppressive compared with their counterparts at other anatomical sites, the molecular mechanism underlying the features of mucosal DCs remains to be understood. We therefore assessed the difference in the frequencies of CD11c+ DCs between Sp and MLNs. MLN CD11c+ DCs displayed significantly or slightly higher levels of B7-DC as well as CD11c and CD103 or B7-H1 than Sp CD11c+ DCs, but similar levels of CD80, CD86, and B7-H2 in WT mice and B7h1−/− mice with the respective targeted gene (Figure 2A and supplemental Figure 4). After stimulation with Cpg ODN, MLN CD11c+ DCs exhibited a higher level of IL-6 than Sp CD11c+ DCs, while they produced a low level of TGF-β1 similar to that in WT mice (Figure 2B).

To visualize the role of the B7 family in the ability of peripheral and gut-associated DCs to convert antigen-specific naive CD4+Foxp3− T cells into CD4+Foxp3+ T cells, we created Foxp3EGFP+CD11.10 mice expressing OVA-specific TCR(KJ1-26 clonotype) and EGFP under the control of the Foxp3 gene (Wang et al20, supplemental Figure 5A-C), and Foxp3EGFP+Rag2−/−CD11.10 mice lacking KJ1-26+CD25+Foxp3EGFP+ nTreg (Sato et al21; supplemental Figure 4D). MLN CD11c+ DCs showed a lesser capacity to activate KJ1-26+CD25+Foxp3EGFP− T cells than Sp CD11c+ DCs in WT mice (Figure 2C). Similar differences were
observed in those of Cδ80/Cδ86−/− mice or B7h2−/− mice although there was a significant or slight reduction of the CD4+ T-cell response compared with that in WT mice (Figure 2D,G). In comparison, a slight difference in the CD4+ T-cell response between Sp Cd11c+ DCs and MLN Cd11c+ DCs was observed in B7h1−/− mice and B7dc−/− mice (Figure 2E-F).

Role of the B7 family expressed on systemic and MLN DCs in iTregs conversion

Recent studies indicate that mucosal DCs efficiently promote the TGF-β1–mediated conversion of CD4+Foxp3− T cells into CD4+Foxp3+ iTregs relative to systemic DCs in vitro,12,24,25 and similar results were obtained when the ability of Sp Cd11c+ DCs and MLN Cd11c+ DCs to induce the antigen-specific generation of KJ1-26+Foxp3EGFP+ Treg cells from KJ1-26+Foxp3EGFP− T cells was compared in the presence of TGF-β1 or TGF-β1 and RA in WT mice (Figure 3). However, the stimulations with IL-6 or CpG ODN markedly inhibited the ability of Sp Cd11c+ DCs and MLN Cd11c+ DCs to induce their generation (supplemental Figure 6). On the other hand, the generation of KJ1-26+Foxp3EGFP+ T cells by Sp Cd11c+ DCs and MLN Cd11c+ DCs was markedly enhanced in Cδ80/Cδ86−/− mice, while the results in B7h2−/− mice were similar to those in WT mice (Figure 3). Importantly, this conversion was dramatically decreased in B7h1−/− mice and B7dc−/− mice (Figure 3). We also addressed the influence of the blockade of PD-1 on the ability of systemic and MLN Cd11c+ DCs to convert antigen-specific naive CD4+ T cells into CD4+Foxp3+ iTregs. The blockade of PD-1 enhanced the capacity of MLN Cd11c+ DCs more than that of Sp Cd11c+ DCs, to activate KJ1-26+CD25+Foxp3EGFP+ T cells, while it had a suppression on the generation of KJ1-26+Foxp3EGFP− T cells by these CD11c+ DCs in WT mice (supplemental Figure 7).

Role of the B7 family expressed on systemic and MLN DCs in Th17 cell conversion

The reciprocal TGF-β1–dependent conversion of naive CD4+ T cells into either CD4+Foxp3+ iTreg or Th17 cells under basal or inflammatory conditions led us to investigate the role of B7 family members in the capacity of systemic and mucosal DCs to drive Th17 cell differentiation (Figure 4). Similar to published reports,12,26 MLN Cd11c+ DCs displayed a reduced capacity to generate antigen-specific IL-17-producing KJ1-26+ T cells from KJ1-26+Foxp3EGFP− T cells in the presence of IL-6 plus TGF-β1 or CpG ODN plus TGF-β1 compared with their splenic counterparts in WT mice. Such Th17 differentiation was enhanced in B7h1−/− mice and B7dc−/− mice or decreased in Cδ80/Cδ86−/− mice and B7h2−/− mice.
Essential roles of B7-H1 and B7-DC in peripheral and mucosal iTreg conversion during oral tolerance

To assess antigen-specific differentiation of CD4+Foxp3+ iTregs from CD4+Foxp3− T cells during the induction of oral tolerance, we adoptively transferred KJ1-26+Foxp3EGFP+ T cells into WT mice, fed these mice OVA, and monitored the generation of KJ1-26+Foxp3EGFP+ iTregs in Sp and MLNs. OVA feeding dramatically generated KJ1-26+Foxp3EGFP+ iTregs in the T-cell areas of MLNs more than those of Sp in WT mice under steady-state conditions (Figure 5A-D). In contrast, oral application of CpG ODN markedly abrogated the generation of these iTregs (Figure 5B-D) as well as oral tolerance (supplemental Figure 8) in OVA-fed WT mice.

To extend our understanding of the role of the B7 family in the antigen-specific development of CD4+Foxp3+ iTregs during the induction of oral tolerance, we examined the generation of KJ1-26+Foxp3EGFP+ iTregs in OVA-fed and nonfed B7−/− mice. The generation of KJ1-26+Foxp3EGFP+ iTregs in Sp and MLNs after oral priming was reduced in Cd80/Cd86−/− mice, but not in B7h2−/− mice, compared with WT mice (Figure 6A-C). On the other hand, the peripheral and mucosal generation of KJ1-26+Foxp3EGFP+ iTregs was severely reduced in B7h1−/− mice compared with WT
mice after oral priming, while such generation was almost completely abolished in $B7dc^{-/-}$ mice (Figure 6A-C).

To further evaluate the antigen-specific regulatory function of CD4+CD25+Foxp3+ iTregs generated after oral priming, CFSE-

Figure 3. Role of B7 family members in the ability of systemic and MLN CD11c+ DCs to generate antigen-specific CD4+Foxp3+ iTregs from CD4+Foxp3+ T cells. Generation of KJ1-26+Foxp3EGFP+ T cells from Sp KJ1-26+Foxp3EGFP+ T cells by Sp or MLN CD11c+ DCs obtained from WT mice and the $B7^{-/-}$ mice in neutral conditions in the presence or absence of TGF-β1 (A-B) or TGF-β1 plus RA (C-D) was analyzed by flow cytometry. Data are represented by a dot plot, and numbers represent the proportion of Foxp3EGFP+ cells among gated CD4+ T cells in each quadrant (A,C) and are the percentage of positive cells (B,D). *P < .01 compared with WT mice. Data are the mean ± SD, and the results are representative of 4 independent experiments.

Figure 4. Role of B7 family members in the ability of systemic and MLN CD11c+ DCs to generate antigen-specific TH17 cells from CD4+Foxp3– T cells. Generation of KJ1-26+IL-17+ T cells from Sp KJ1-26+Foxp3EGFP+ T cells by Sp (A-B) or MLN (A,C) CD11c+ DCs obtained from WT mice and the $B7^{-/-}$ mice in neutral conditions in the presence or absence of TGF-β1, IL-6 plus TGF-β1, or CpG ODN plus TGF-β1 was analyzed by flow cytometry. Data are represented by a dot plot, and numbers represent the proportion of IL-17+ cells among gated CD4+ T cells in each quadrant (A) and are the percentage of positive cells (B-C). *P < .01 compared with WT mice. Data are the mean ± SD, and the results are representative of 4 independent experiments.
labeled KJ-1-26 Foxp3− T cells were adoptively transferred into WT mice with or without CD4+CD25+ T cells obtained from OVA-fed and nonfed Foxp3EGFP DO11.10 mice, WT mice and B7−/− mice, and the dilution of CFSE-labeled KJ-1-26+ T cells as an indicator of in vivo proliferation was analyzed after systemic injection of OVA (Figure 6D-F). We observed that more than 90% of CD4+CD25+ T cells obtained from each group of mice expressed Foxp3 protein (data not shown). The transfer of CD4+CD25+ T cells obtained from nonfed WT mice slightly suppressed the antigen-specific division of KJ-1-26+ T cells, whereas those obtained from nonfed B7h1−/− mice and B7dc−/− mice had little or no effect on this response. In contrast, the transfer of CD4+CD25+ T cells obtained from OVA-fed WT mice as well as nonfed Foxp3EGFP DO11.10 mice markedly abrogated the antigen-specific proliferative response of KJ-1-26+ T cells, while those obtained from OVA-fed B7h1−/− mice and B7dc−/− mice slightly enhanced this response.

Discussion

We show here the possible prerequisite role of B7-H1 and B7-DC expressed on MLN DCs in both the damping of antigen-specific CD4+ T-cell responses and induction of active immune regulation mediated through the de novo conversion of antigen-specific naive CD4+Foxp3− T cells into CD4+Foxp3+ iTregs under steady-state conditions, thereby leading to the establishment of oral tolerance.

Despite an extensive number of studies on the impact of the B7-DC28 family on peripheral immune response,8,9 the roles of these molecules in oral tolerance remain elusive. We showed that the antigen-specific immune response was enhanced in B7h1−/− mice and B7dc−/− mice or decreased in Cd80/Cd86−/− mice and B7h2−/− mice compared with WT mice after systemic immunization with antigen. Indeed, the ability of Sp CD11c+ DCs to activate antigen-specific naive CD4+ T cells was also higher in B7h1−/− mice and B7dc−/− mice or lower in Cd80/Cd86−/− mice and B7h2−/− mice than it was in WT mice. Therefore, B7-H1 and B7-DC serve as negative regulators, whereas CD80/CD86 and B7-H2 are positive costimulators for peripheral immune response. On the other hand, antigen feeding before systemic immunization inhibited the antigen-specific immune response in WT mice as well as Cd80/Cd86−/− mice and B7h2−/− mice, whereas this treatment failed to abrogate the response in B7h1−/− mice and B7dc−/− mice. Therefore, B7-H1 and B7-DC could be essential for establishing oral tolerance.

CD80/Cd86;CD28 interactions reportedly regulate the development of self-reactive thymic-derived CD4+Foxp3+ iTregs on the C57BL/6 background.27 We showed that the frequency and the absolute number of CD4+Foxp3+ iTregs in Sp and MLNs was lower in Cd80/Cd86−/− mice and B7h2−/− mice than in WT mice,

Figure 5. Antigen-specific de novo generation of CD4+Foxp3+ iTregs from CD4+Foxp3- T cells in Sp and MLNs during the induction of oral tolerance. (A-D) Sp KJ-1-26 Foxp3EGFP T cells were transferred into WT mice (5 per group) that had been treated with CpG ODN (B-D), and the animals were subsequently fed PBS (none) or OVA protein the day after the adoptive transfer. Image acquisition information: BIOREVO BZ-9000 fluorescence microscope (KEYENCE); immunofluorescence, 10x, 10x/0.25 objective lenses; room temperature; no imaging medium; Alexa Fluor 488, R-phycocerythrin, Alexa Fluor 647, DAPI fluorochromes; BZ-II Analyzer acquisition software (KEYENCE); JPEG, Preview 3.0.9 (Apple Inc). (B-D) Expression of Foxp3EGFP among gated KJ-1-26+ T cells in Sp (B-C) and MLNs (B-D) on day 11 after the adoptive transfer was analyzed by flow cytometry. Data are represented by a dot plot, and numbers represent the proportion of Foxp3+ T cells obtained from OV A-fed WT mice as well as T cells obtained from nonfed WT mice slightly higher in CD4+Foxp3+ iTregs under steady-state conditions, thereby leading to the establishment of oral tolerance.

Discussion

We show here the possible prerequisite role of B7-H1 and B7-DC expressed on MLN DCs in both the damping of antigen-specific CD4+ T-cell responses and induction of active immune regulation mediated through the de novo conversion of antigen-specific naive CD4+Foxp3− T cells into CD4+Foxp3+ iTregs under steady-state conditions, thereby leading to the establishment of oral tolerance.

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CD80/Cd86;CD28 interactions reportedly regulate the development of self-reactive thymic-derived CD4+Foxp3+ iTregs on the C57BL/6 background.27 We showed that the frequency and the absolute number of CD4+Foxp3+ iTregs in Sp and MLNs was lower in Cd80/Cd86−/− mice and B7h2−/− mice than in WT mice,
whereas it was similar in B7hl-/- mice or B7dc-/- mice with the BALB/c background. Previous studies suggest that IL-2 signaling is required for maintaining the homeostasis of CD4+Foxp3+ nTregs,6,27,28 and CD80/CD8629 and B7-H219 expressed on APCs are crucial for IL-2 production by CD4+ T cells. Therefore, costimulation through CD80/CD86;CD28 and B7-H2;ICOS is necessary for thymic development and IL-2-mediated expansion of CD4+Foxp3+ nTregs for maintaining their homeostasis in peripheral and intestinal mucosa. In contrast, antigen feeding enhanced the frequency of CD4+Foxp3+ Tregs in Sp and MLNs in WT mice as well as

Figure 6. Role of the B7 family in antigen-specific de novo generation of CD4+Foxp3+ iTregs from CD4+Foxp3- T cells in Sp and MLNs. (A-C) Sp KJ1-26 + Foxp3EGFP- T cells were transferred into WT mice and B7hl-/- mice (5 per group), and the animals were subsequently fed PBS (none) or OVA protein the day after the adoptive transfer. Expression of Foxp3EGFP among gated KJ1-26+ T cells in Sp (A-B) and MLNs (A,C) on day 11 after the adoptive transfer was analyzed by flow cytometry. Data are represented by a dot plot, and numbers represent the proportion of Foxp3EGFP+ cells among gated KJ1-26+ T cells in each quadrant (A) and are the percentage of positive cells (B-C). *P < .01 compared with WT mice. Data are the mean ± SD, and the results are representative of 4 independent experiments. (D-F) CFSE-labeled Sp Rag2-/-KJ1-26+ T cells (3 x 10^6/mouse) were transferred into WT mice (5 per group) with or without Sp CD4+CD25+ T cells (10^6/mouse) obtained from Foxp3EGFPDO11.10 mice (D,F), WT mice (E,F), and B7hl-/- mice (E-F) that had been fed PBS (none) or OVA protein, and the animals were subsequently injected with OVA protein the day after the adoptive transfer. CFSE dilution among gated KJ1-26+ T cells on day 3 after the injection with OVA protein was analyzed by flow cytometry. Data are represented by a dot plot, and numbers represent the proportion of CFSE dilution among gated KJ1-26+ T cells in each quadrant (D-E) and are the percentage of dividing cells (F). *P < .01 compared with Rag2-/-KJ1-26+ T cells plus OVA. Data are the mean ± SD, and the results are representative of 4 independent experiments.
CD80/Cd86−/− mice and B7h2−/− mice, whereas it had little or no effect in B7hl−/− mice and B7dc−/− mice. Thus, B7-H1 and B7-DC could be required for the antigen-specific generation and expansion of CD4+Foxp3+ Treg cells in peripheral and intestinal mucosa during the induction of oral tolerance.

Analysis of WT mice and B7−/− mice revealed that the increased frequency of CD4+Foxp3+ Treg cells in Sp and MLNs was clearly associated with the establishment of oral tolerance. In addition, the depletion of CD4+Foxp3+ Treg cells in Sp and MLNs resulted in a failure to establish oral tolerance in WT mice. Therefore, the antigen-specific dominant tolerance involving CD4+Foxp3+ Treg cells, rather than the recessive tolerance mediated by anergy, exhaustion, and deletion of antigen-specific CD4+ T cells could mainly contribute to the B7-H1− and B7-DC-mediated establishment of oral tolerance.

We showed that MLN CD11c+ DCs exhibited higher levels of B7-H1 and B7-DC, but not other B7 family members, and a lesser capacity to activate antigen-specific naive CD4+ T cells than Sp CD11c+ DCs in WT mice. In addition, a similar capacity to activate antigen-specific naive CD4+ T cells between Sp CD11c+ DCs and MLN CD11c+ DCs was observed in B7hl−/− mice and B7dc−/− mice, but not in CD80/Cd86−/− mice or B7h2−/− mice. Therefore, the dominant expression of B7-H1 and B7-DC could be responsible for the suppressive effect of MLN CD11c+ DCs on the CD4+ T cell-response. The reason why MLN CD11c+ DCs showed the prominent expression of B7-H1 and B7-DC as well as I-AL-E and CD103 remains unclear, but constitutive exposure to commensal bacteria in an intestinal environment associated with a large amount of TGF-β30 may account for the up-regulated expression.

Much attention has been paid to cytokine-mediated differentiation into CD4+Foxp3+ iTregs and Tikh7 cells, but far less is known about the role of the B7-CD28 family in the program for the development of these cells. In WT mice, MLN CD11c+ DCs showed a greater capacity to induce the TGF-β1−mediated antigen-specific conversion of CD4+Foxp3+ T cells into CD4+Foxp3+ iTregs than did Sp CD11c+ DCs in vitro. On the other hand, B7hl−/−CD11c+ DCs and B7dc−/−CD11c+ DCs impaired the ability to generate CD4+Foxp3+ iTregs, whereas CD80/Cd86−/−CD11c+ DCs enhanced this capacity compared with WT CD11c+ DCs. In addition, the blockade of PD-1 was also effective in abrogating the capacity of Sp and MLN CD11c+ DCs to generate CD4+Foxp3+ iTregs in WT mice, suggesting that the interaction of B7-H1 and B7-DC with PD-1 play a crucial role in the conversion of CD4+Foxp3+ T cells into CD4+Foxp3+ iTregs, whereas costimulation through CD80/Cd86/Cd28 abrogates this conversion. Collectively, the dominant expression of B7-H1 and B7-DC, in addition to a high level of RA,12,24,25 could be involved in the superior capacity of MLN CD11c+ DCs to generate CD4+Foxp3+ iTregs.

Consistent with previous reports,12,26 CD11c+ DCs required IL-6 plus TGF-β1 for the generation of Tikh7 cells from naive CD4+ T cells in vitro. In addition, Sp CD11c+ DCs exhibited a greater capacity to induce the development of Tikh7 cells than did MLN CD11c+ DCs under inflammatory conditions, but were less effective for the production of IL-6 than MLN CD11c+ DCs. On the other hand, B7hl−/−CD11c+ DCs and B7dc−/−CD11c+ DCs enhanced the ability to generate Tikh7 cells, whereas CD80/Cd86−/−CD11c+ DCs and B7h2−/−CD11c+ DCs reduced this capacity compared with WT CD11c+ DCs, indicating that B7-H1 and B7-DC suppresses, whereas CD80/Cd86 and B7-H2 participate in, Tikh7 cell differentiation. Thus, the higher expression of B7-H1 and B7-DC, regardless of the greater production of IL-6, could account for the reduced capacity of MLN CD11c+ DCs to prime antigen-specific naive CD4+ T cells for differentiation into Tikh7 cells compared with systemic CD11c+ DCs.

Our results obtained using an adoptive transfer system clearly showed that antigen feeding more effectively induced the antigen-specific generation of CD4+Foxp3+ iTregs from CD4+Foxp3− T cells in MLNs than Sp in WT mice. It has been reported that antigen transport from LP via afferent lymphatics into MLNs is necessary for oral tolerance, although antigens entering the LP also disseminate via the circulation in minute amounts into peripheral tissues.2 These observations support the hypothesis that MLNs are primary sites for de novo generation of CD4+Foxp3+ iTregs after antigen feeding, and CD4+Foxp3+ iTregs that have migrated from MLNs into the circulation as well as those generated in peripheral tissues suppress systemic antigen-specific immune responses upon a subsequent systemic antigen challenge, resulting in the establishment of oral tolerance.

Previous studies suggest that the program for the generation and homeostasis of CD4+Foxp3+ iTregs requires TCR stimulation and cytokines including TGF-β and IL-2 in vivo,6 but the precise molecular mechanism involved remains unclear. We showed that the de novo generation of CD4+Foxp3+ iTregs in Sp and MLNs after oral priming was impaired in B7dc−/− mice more than in B7hl−/− mice compared with WT mice, and these phenomena were accompanied by a failure to establish oral tolerance. Therefore, these results suggest that B7-DC and B7-H1 are necessary for the de novo generation of CD4+Foxp3+ iTregs in vivo. On the other hand, CD80/Cd86−/− mice had fewer of these CD4+Foxp3+ iTregs than WT mice, and the discrepancy regarding the influence of CD80/Cd86 on the development of CD4+Foxp3+ iTregs between in vivo and in vitro might be due to the inefficiency of IL-2−mediated expansion.6,27 Collectively, these results suggest that B7-DC and B7-H1 play a crucial role in the differentiation of antigen-specific CD4+Foxp3− T cells into CD4+Foxp3+ iTregs, while CD80/Cd86 participate in their IL-2−mediated expansion during the establishment of oral tolerance.

It has been suggested that oral tolerance is induced in the absence of CD4+Foxp3+ nTregs, and the establishment of oral tolerance correlates with the de novo induction of antigen-specific CD4+Foxp3+ iTregs.31 We showed that CD4+CD25+Foxp3+ T cells obtained from antigen-fed WT mice showed a more potent suppression on the activation of antigen-specific CD4+ T cells than those obtained from nonfed WT mice, implying that CD4+CD25+Foxp3+ iTregs generated after oral priming rather than CD4+CD25+Foxp3+ nTregs play a main role in the suppression of antigen-specific response of CD4+ T cells. On the other hand, CD4+CD25+Foxp3+ T cells obtained from antigen-fed B7hl−/− mice and B7dc−/− mice enhanced the antigen-specific CD4+ T-cell response, whereas those obtained from nonfed B7hl−/− mice and B7dc−/− mice failed to suppress this response. These findings led us to hypothesize that B7-H1 and B7-DC are required for the de novo generation of antigen-specific CD4+CD25+Foxp3+ iTregs and the maintenance of their function after oral priming that leads to the inhibition of antigen-specific immune response, to establish oral tolerance.

Series of studies have shown that B7-H1 is constitutively expressed on APCs and T cells as well as nonhematopoietic cells, whereas B7-DCs is expressed primarily on a small portion of DCs in peripheral tissues under steady-state conditions in vivo.8,9,22 We showed that B7-H1 and B7-DC were preferentially expressed on MLN CD11c+ DCs compared with Sp CD11c+ DCs. On the other hand, it has been reported that CCR7-dependent migration of DCs
from the intestine to MLNs after antigen uptake is obligatory for oral tolerance. Therefore, these phenomena imply that MLN DCs induce CD4+Foxp3+ iTregs conversion but also enhances their ability to generate inflammatory T_h17 cells, leading to a blockade of oral tolerance and a promotion of inflammatory intestinal immune disorders.

Mucosal tolerance is suggest to prevent pathologic reactions against environmental and food antigens, and its failure results in exacerbated inflammation typical of food allergies and asthma. Although the precise role of B7 family members in the initiation and the promotion of human allergic diseases remains unclear, but the expression balance between CD80/CD86 and CD89/CD80, and the activation of antigen-presenting cells induce CD4+Foxp3+ iTregs conversion 


t is mediated antigen-specific inhibition of CD4+Foxp3+ iTregs by MLN DCs through B7 family members.


the expression balance between CD80/CD86 and B7-H1 versus B7-DC on the mucosal tissues may be involved in these immunopathologies. Further study will be needed to examine this possibility. In addition, recent study has shown that murine B7-H1-human IgG1 Fc fusion protein-coupled beads efficiently promoted CD4+Foxp3+ iTregs conversion. Therefore, oral administration of human B7-H1 and B7-DC-human IgG1 Fc fusion protein-coupled biodegradable nanoparticles may provide an advantageous means of the intervention for human allergic diseases. On the other hand, it has been suggested that mucosal tolerance is a limiting factor in successful oral immunization with vaccines to induce the protective immune responses against intestinal infectious diseases. Therefore, the intervention of B7-H1- and B7-DC-mediated pathway by oral application of their blocking mAbs might be a potential strategy to improve the efficacy of human antimi crobial oral vaccine.

In conclusion, our findings suggest that the fine-tuning of iTreg/Teff cell equilibrium by MLN DCs through B7 family members is instrumental in the maintenance of intestinal immune homeostasis, should promote understanding of the nature of intestinal immune pathophysiology and may open new avenues for exploring therapeutic strategies to mediate oral tolerance for immune disorders, and prevent tolerance to oral vaccines against infectious diseases.

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Authorship

Contribution: Katsuki Sato designed the research project, analyzed data and wrote the paper; T.F., H. Takagi, Y.S., Kori Sato, K.E., and H. Taya performed the experiments; and T.S., L.C., C.D., M.A., H.Y., and B.M. contributed vital new reagents or analytical tools.

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Crucial roles of B7-H1 and B7-DC expressed on mesenteric lymph node dendritic cells in the generation of antigen-specific CD4+Foxp3+ regulatory T cells in the establishment of oral tolerance

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