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β -Glucan Curdlan Induces IL-10–Producing CD4⁺ T Cells and Inhibits Allergic Airway Inflammation

Saki Kawashima,¹ Koichi Hirose,¹ Arifumi Iwata, Kentaro Takahashi, Ayako Ohkubo, Tomohiro Tamachi, Kei Ikeda, Shin-ichiro Kagami, and Hiroshi Nakajima

A number of studies have suggested a correlation between a decreased incidence in infectious diseases and an increased incidence of allergic diseases, including asthma. Although several pathogen-derived products have been shown to possess therapeutic potential for allergic diseases, it remains largely unknown whether β -glucan, a cell wall component of a variety of fungi, yeasts, and bacteria, has a regulatory potential for allergic diseases. In this study, we examined the effect of curdlan, a linear β -(1-3)-glucan, on the development of allergic airway inflammation. We found that i.p. injection of curdlan significantly inhibited Ag-induced eosinophil recruitment and Th2 cytokine production in the airways. The activation of CD4⁺ T cells in the presence of curdlan induced IL-10–producing CD4⁺ T cells with high levels of c-Maf expression. Curdlan-induced development of IL-10–producing CD4⁺ T cells required the presence of APCs and ICOS/ICOS ligand interaction. Curdlan-induced development of IL-10–producing CD4⁺ T cells also required intrinsic expression of STAT6. Furthermore, the transfer of Ag-specific CD4⁺ T cells that were stimulated in the presence of curdlan inhibited Ag-induced eosinophil recruitment into the airways. Taken together, these results suggest that curdlan is capable of inducing IL-10–producing CD4⁺ T cells and inhibiting the development of eosinophilic airway inflammation, underscoring the therapeutic potential of curdlan for allergic diseases. *The Journal of Immunology*, 2012, 189: 000–000.

Asthma is characterized by chronic airway inflammation with intense eosinophil infiltration, mucus hypersecretion, and airway hyperresponsiveness against a wide variety of stimuli (1, 2). It is well established that these features are mainly mediated by Ag-specific Th2 cells and their cytokines, including IL-4, IL-5, IL-9, and IL-13 (1, 2). Additionally, several epidemiologic studies suggest that lack of early exposure against infectious agents is associated with an increased incidence of allergic diseases, including asthma (3–6).

Consistent with the inverse correlation between infectious diseases and allergy, a number of mouse and human studies have proposed the therapeutic potential of viral and bacterial products for allergic diseases. For example, vaccination of the bacillus Calmette-Guérin–attenuated strain of *Mycobacterium bovis* has been shown to reduce the levels of serum IgE and suppress the development of allergic diseases (7–9). Immunotherapy using DNA containing the CpG motif has also been reported to suppress Ag-specific IgE and show a long-lasting beneficial effect after

immunotherapy (10). Additionally, vaccination with heat-killed *Listeria monocytogenes* inhibits Ag-induced allergic inflammation through the conversion of Th2 cells to Th1 cells and the induction of IL-10–producing CD4⁺ T cells (11).

β -glucans are microorganism-associated molecular patterns present in a wide variety of fungal and bacterial cell walls, seaweeds, and edible mushrooms (12). Although there is contradictory evidence regarding the effects of β -glucan on CD4⁺ T cell differentiation (13–15), a number of studies suggest the therapeutic potential of β -glucan for autoimmune and allergic diseases. For example, Karumuthil-Melethil et al. (16) have shown that i.p. injection of zymosan, a β -glucan prepared from cell wall of *Saccharomyces cerevisiae*, inhibits the development of diabetes in NOD mice. Additionally, superfine dispersed β -(1-3)-glucan, which is prepared from the edible mushroom *Lentinus edodes*, has been shown to reduce the symptoms of allergic rhinitis (17). However, therapeutic potential of β -glucans for allergic airway inflammation and the underlying mechanisms remain largely unknown.

In this study, we examined the effect of curdlan, a linear β -(1-3)-glucan derived from *Alcaligenes faecalis*, on allergic airway inflammation using a murine model of asthma. Our results elucidate that curdlan induces IL-10–producing CD4⁺ T cells and inhibits Ag-induced eosinophil recruitment into the airways.

Materials and Methods

Mice

OVA-specific DO11.10 TCR transgenic mice (18), T-bet–deficient (T-bet^{−/−}) mice (19), and STAT6-deficient (STAT6^{−/−}) mice (20) were backcrossed for 10 generations onto BALB/c mice (Charles River Laboratories, Kanagawa, Japan). All mice were housed in microisolator cages under specific pathogen-free conditions, and animal procedures used in this study were approved by the Chiba University Animal Care and Use Committee.

Preparation of curdlan

Curdlan was purchased from Wako Pure Chemical Industries (Osaka, Japan). To linearize curdlan, it was once solubilized in 0.15 N NaOH solution

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Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; DC, dendritic cell; ICOSL, ICOS ligand; ROR, retinoic acid–related orphan receptor; Treg, regulatory T cell; WT, wild-type.

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and the culture medium was added to the solution to neutralize pH as described elsewhere (21).

Ag-induced allergic airway inflammation

BALB/c mice (age 6–8 wk) were sensitized and challenged with OVA as described previously (22). In brief, BALB/c mice were immunized i.p. twice with 10 µg OVA in 4 mg aluminum hydroxide at a 2-wk interval, and 2 wk after the second immunization the sensitized mice were administered aerosolized OVA (50 mg/ml) for 20 min through a DeVilbiss 646 nebulizer (DeVilbiss, Somerset, PA). Where indicated, mice were injected i.p. with indicated amounts of curdlan suspended in PBS or PBS alone at 24 h before OVA inhalation (except for Supplemental Fig. 1). The numbers of eosinophils, neutrophils, and lymphocytes recovered from bronchoalveolar lavage fluid (BALF) were evaluated at 48 h after the OVA inhalation, as described previously (23). Lung sections (3 µm thick) were stained with H&E according to standard protocols.

IL-10 production of lung CD4⁺ T cells

Lung was cut into small fragments and digested with collagenase A solution (1 mg/ml; Roche, Indianapolis, IN) in RPMI 1640 medium containing 10% FCS for 40 min at 37°C with continuous agitation. After filtering through 0.07-mm nylon mesh to remove aggregates, viable lymphocytes were enriched from the cell suspension using Lympholyte-M solution (Cedarlane Laboratories, Burlington, ON, Canada) according to the manufacturer's instructions. After washing, isolated lymphocytes were stimulated with PMA (20 ng/ml; Calbiochem, San Diego, CA) plus ionomycin (1 µg/ml; Calbiochem) at 37°C for 4 h in the presence of monensin (2 µM; Sigma-Aldrich, St. Louis, MO). After surface staining with anti-CD4-FITC (BD Biosciences, San Diego, CA), cells were fixed, permeabilized, and stained with anti-IL-10-allophycocyanin (JES5-16E3; BD Biosciences). After washing, cells were analyzed on a FACSCalibur using CELLQuest Pro software (BD Biosciences).

Purification of splenic dendritic cells

Splenic CD11c⁺ dendritic cells (DCs) were prepared as described previously (24). In brief, spleens were cut into small fragments and then digested with collagenase A (0.5 mg/ml) for 10 min at 37°C with continuous agitation. After digested fragments were filtered through a stainless steel sieve, low-density cells were isolated by using OptiPrep reagent (Axis-Shield, Oslo, Norway). Low-density cells were then stained with anti-CD11c-FITC and after washing with PBS, FITC-stained cells were positively collected using anti-FITC microbeads (Miltenyi Biotec, Sunnyvale, CA) according to the manufacturer's instructions. The resultant cells were >95% pure CD11c⁺ cells by FACS analysis.

Ag-induced T cell differentiation

Splenocytes (1×10^6 /ml) from DO11.10 mice were stimulated with OVA_{323–339} peptide (5 µg/ml) in 48-well microtiter plates at 37°C. Where indicated, IL-12 (10 ng/ml) was added to polarize toward Th1 cells (Th1 conditions), IL-4 (10 ng/ml) was added to polarize toward Th2 cells (Th2 conditions), IL-6 (20 ng/ml) and TGF-β (2 ng/ml) were added to polarize toward Th17 cells (Th17 conditions), and TGF-β (2 ng/ml) and IL-2 (10 ng/ml) were added to polarize toward regulatory T cells (Treg; Treg conditions).

Effect of curdlan on T cell differentiation

Splenocytes from DO11.10 mice, T-bet^{−/−} DO11.10 mice, and STAT6^{−/−} DO11.10 mice were stimulated with OVA peptide as described above in the presence or absence of indicated amounts of curdlan. In some experiments, anti-Dectin-1 mAb (2A11; 5 µg/ml) (25), anti-ICOS ligand (ICOSL) Ab (HK5.3; 10 µg/ml), or isotype-matched control Ab (rat IgG2a, 10 µg/ml; BD Biosciences) was added to the culture.

Adoptive transfer experiments for Ag-induced airway inflammation

Splenocytes from DO11.10 mice were stimulated with OVA peptide in the presence or absence of curdlan as described above. After washing, live CD4⁺ T cells were enriched from the culture by using Lympholyte-M according to the manufacturer's instructions and transferred i.v. into OVA-sensitized BALB/c mice (2×10^6 cells/mouse). Twenty-four hours later, the mice were challenged with OVA inhalation. The numbers of eosinophils, neutrophils, and lymphocytes recovered from the BALF, the levels of IL-5, IL-13, and IL-17A in the BALF, and histological findings of the lung were evaluated at 48 h after OVA inhalation.

Cytokine assay

The amounts of IL-10 and IL-4 were determined by ELISA kits from BD Biosciences. The amounts of IFN-γ and IL-17A were determined by ELISA kits from R&D Systems (Minneapolis, MN).

Intracellular staining

Cells were washed and restimulated with PMA (20 ng/ml) plus ionomycin (1 µg/ml) at 37°C for 4 h in the presence of monensin. The expression levels of cytokines and Foxp3 were evaluated by intracellular staining for IFN-γ, IL-4, IL-17A, IL-10, and Foxp3 as described previously (26).

Real-time PCR analysis

Live CD4⁺ T cells were isolated from the culture by using Lympholyte-M and a CD4⁺ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. The expression levels of T-bet, GATA3, retinoic acid-related orphan receptor (ROR)γt, Foxp3, and c-Maf mRNA were determined by real-time PCR using a SYBR Green reagent (Power SYBR Green PCR Master Mix; Applied Biosystems, Foster City, CA) with a standard protocol on an ABI Prism 7300 instrument (Applied Biosystems). The expression levels of each mRNA were normalized on the levels of β-actin.

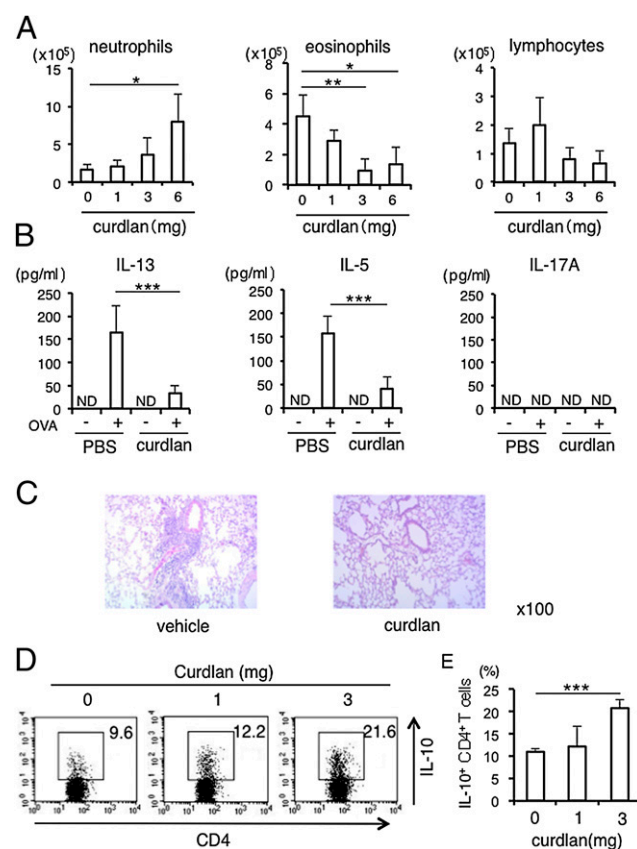


FIGURE 1. Curdlan inhibits Ag-induced eosinophil recruitment into the airways. (A–E) OVA-sensitized BALB/c mice were injected i.p. with the indicated dose of curdlan suspension or vehicle (PBS) as described in *Materials and Methods*. Twenty-four hours after the injection, mice were challenged with OVA inhalation. (A) The numbers of neutrophils, eosinophils, and lymphocytes in the BALF were evaluated at 48 h after OVA inhalation. Data are means $\pm$ SD for four mice in each group. * p < 0.05, ** p < 0.01. (B) The levels of IL-5, IL-13, and IL-17A in the BALF were measured by ELISA at 48 h after OVA inhalation. Data are means $\pm$ SD for four mice in each group. *** p < 0.005. (C) Histological analysis of the lung (H&E staining) was performed at 48 h after OVA inhalation. Representative photomicrographs of lung sections are shown (n = 4, each). Original magnification $\times$ 100. (D and E) Intracellular IL-10 staining of lung CD4⁺ T cells was performed at 48 h after OVA inhalation as described in *Materials and Methods*. Representative FACS profiles of IL-10 staining on CD4⁺ T cells (D) and the frequency of IL-10⁺ cells in CD4⁺ T cells (E) are shown (n = 5). *** p < 0.005.

Data analysis

Data are summarized as means $\pm$ SD. The statistical analysis of the results was performed by the unpaired *t* test. A *p* value <0.05 was considered significant.

Results

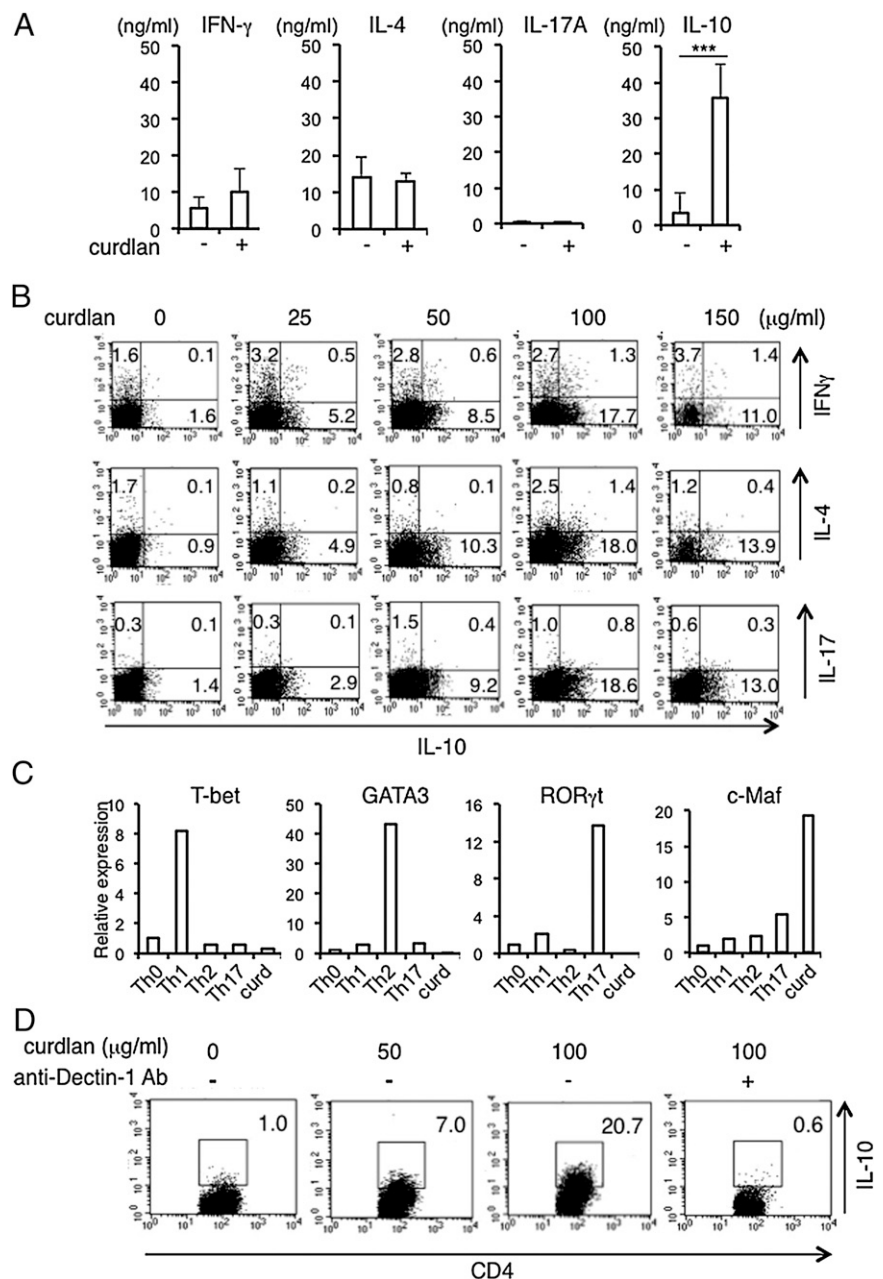
Intraperitoneal injection of curdlan inhibits Ag-induced eosinophil recruitment into the airways

To investigate the therapeutic potential of a linear β -(1-3)-glucan curdlan on Ag-induced allergic diseases, we first examined the effects of curdlan on Ag-induced airway inflammation in a murine model of asthma. BALB/c mice were immunized twice with OVA at a 2-wk interval, and 2 wk after the second immunization, mice were injected i.p. with a suspension of curdlan or vehicle (PBS). Twenty-four hours later, mice were challenged with OVA inhalation and airway inflammation was evaluated at 48 h after OVA inhalation. As shown in Fig. 1A (middle panel), 3 or 6 mg but not 1 mg curdlan significantly inhibited eosinophil recruitment into

the airways ($n = 4$ in each group; $p < 0.01$ for 3 mg and $p < 0.05$ for 6 mg). Alternatively, 6 mg curdlan significantly enhanced neutrophil recruitment into the airways ($n = 4$, $p < 0.05$) (Fig. 1A, left panel). Similarly, injection of 3 mg curdlan at 48 h before OVA inhalation inhibited eosinophil recruitment into the airways, whereas injection of curdlan at 24 h after OVA inhalation did not inhibit eosinophil recruitment into the airways (Supplemental Fig. 1). After the optimization process, experimental conditions with injection of 3 mg curdlan at 24 h before OVA inhalation were used in subsequent experiments.

We next evaluated the effect of curdlan on cytokine production in the airways. As shown in Fig. 2B, curdlan decreased the levels of IL-13 and IL-5 in the BALF ($n = 4$, $p < 0.005$) (Fig. 1B), consistent with the reduced eosinophil infiltration. Although previous reports have suggested the ability of curdlan to induce Th17 cell differentiation (13), IL-17A was undetectable in the BALF even in curdlan-injected mice in our systems (Fig. 1B). Histological analysis of the lungs revealed that Ag-induced peribronchiolar and

FIGURE 2. Curdlan induces IL-10-producing CD4⁺ T cells in a Dectin-1-dependent mechanism. **(A)** Splenocytes from DO11.10 mice were stimulated with OVA peptide in the presence or absence of curdlan (100 μ g/ml) for 5 d. After washing with PBS, live CD4⁺ T cells were collected and restimulated with anti-CD3 Ab plus anti-CD28 Ab (anti-CD3/CD28) for 24 h. The amounts of indicated cytokines in the culture supernatants were measured by ELISA. Data are means $\pm$ SD ($n = 5$). *** $p < 0.005$. **(B)** Splenocytes from DO11.10 mice were stimulated with OVA peptide in the presence of indicated amounts of curdlan for 5 d. After washing, cells were restimulated with PMA plus ionomycin for 4 h and intracellular staining for IL-10, IFN- γ , IL-4, and IL-17A on CD4⁺ T cells was performed. Shown are representative FACS profiles from three independent experiments. **(C)** Splenocytes from DO11.10 mice were stimulated with OVA peptide stimulated in the presence of curdlan (100 μ g/ml) for 5 d. Live CD4⁺ T cells were collected and mRNA levels of T-bet, GATA3, ROR γ t, and c-Maf were evaluated by real-time PCR analysis. CD4⁺ T cells cultured under Th1, Th2, and Th17 conditions were also subjected to real-time PCR analysis as controls. Shown are representative data of relative expression of each transcription factor in each culture condition to those in Th0 conditions ($n = 3$). **(D)** Splenocytes from DO11.10 mice were stimulated with OVA peptide in the presence of indicated amounts of curdlan with or without anti-Dectin-1 mAb for 5 d. After washing, cells were restimulated with PMA plus ionomycin for 4 h and intracellular staining for IL-10 on CD4⁺ T cells was performed. Results shown are representative of three independent experiments.



perivascular inflammation, which mainly consisted of eosinophils and lymphocytes, was attenuated in curdlan-injected mice (Fig. 1C). These results suggest that curdlan inhibits Ag-induced eosinophil recruitment into the airways by suppressing Th2 cytokine production.

Because it has been reported that inhibitory effects of zymosan, a fungal wall component containing β -glucan, on the development of autoimmune diabetes are mediated by IL-10-producing CD4⁺ T cells (16), we next examined whether IL-10-producing CD4⁺ T cells were accumulated in the lung by curdlan injection. Intracellular analysis on CD4⁺ T cells revealed that the number of IL-10-producing CD4⁺ T cells in the lung was increased by curdlan injection (vehicle, $10.8 \pm 0.9\%$ versus curdlan (3 mg), $20.8 \pm 1.9\%$; $n = 5$, $p < 0.005$) (Fig. 1D, 1E). These results suggest that increased accumulation of IL-10-producing CD4⁺ T cells might be involved in curdlan-mediated inhibition of eosinophilic airway inflammation.

Curdlan induces IL-10 production in CD4⁺ T cells in a Dectin-1-dependent manner

To address the mechanism underlying the curdlan-mediated induction of IL-10-producing CD4⁺ T cells, we next examined the effect of curdlan on Ag-induced CD4⁺ T cell differentiation. Single-cell suspension of splenocytes from OVA-specific DO11.10 mice was stimulated with OVA peptide without any exogenous cytokines or anticytokine Abs in the presence or absence of curdlan for 5 d. CD4⁺ T cells were restimulated with anti-CD3 Ab plus anti-CD28 Ab for 24 h and the levels of IFN- γ , IL-4, IL-17A, and IL-10 in the culture supernatants were measured by ELISA. Whereas the levels of IFN- γ , IL-4, and IL-17A in the supernatants of splenocytes stimulated in the presence of curdlan were comparable to those stimulated in the absence of curdlan, the levels of IL-10 were significantly increased in the supernatants of splenocytes stimulated in the presence of curdlan ($n = 5$, $p < 0.005$) (Fig. 2A).

Given that several subpopulations of CD4⁺ T cells including Th1 cells (27, 28), Th2 cells (29), and Th17 cells (30) have been shown to produce IL-10, we next examined whether curdlan-induced IL-10-producing CD4⁺ T cells also produce IFN- γ , IL-4, or IL-17A at single cell levels. Consistent with data shown in Fig. 2A, curdlan induced the development of IL-10-producing CD4⁺ T cells, and the maximum efficiency of curdlan was observed at 100 μ g/ml (Fig. 2B). Although a small portion of curdlan-induced IL-10-producing CD4⁺ T cells produced IFN- γ , IL-4, or IL-17, most of them did not produce these cytokines (Fig. 2B). We also examined the expression levels of T-bet, GATA3, and ROR γ t, master regulators for Th1 cells, Th2 cells, and Th17 cells, respectively, in CD4⁺ T cells stimulated in the presence of curdlan. However, these cells did not express T-bet, GATA3, or ROR γ t (Fig. 2C), further supporting the notion that these IL-10-producing CD4⁺ T cells are different from Th1 cells, Th2 cells, or Th17 cells. Because transcription factor c-Maf has been reported to be involved in IL-10 production (31, 32), we also examined the expression of c-Maf mRNA in CD4⁺ T cells stimulated in the presence of curdlan. Intriguingly, CD4⁺ T cells stimulated in the presence of curdlan expressed higher levels of c-Maf than Th2 cells and Th17 cells (Fig. 2C), in which c-Maf has been shown to be highly expressed (32, 33).

It has been shown that myeloid-specific C-type lectin receptor Dectin-1 is a receptor for curdlan (13). To exclude the possibility that a contaminant from the preparation of curdlan rather than curdlan itself induces the development of IL-10-producing CD4⁺ T cells, we next examined whether curdlan-induced development of IL-10-producing CD4⁺ T cells depended on Dectin-1. As shown in Fig. 2D, curdlan-induced IL-10 production in CD4⁺

T cells was inhibited by anti-Dectin-1 mAb. Taken together, these results indicate that curdlan induces IL-10-producing CD4⁺ T cells, which are distinct from Th1 cells, Th2 cells, and Th17 cells, in a Dectin-1-dependent manner.

Curdlan-induced IL-10-producing CD4⁺ T cells are distinct from Foxp3⁺ regulatory T cells

It has been demonstrated that Foxp3⁺ Tregs produce large amounts of IL-10 (34). To assess the disparity between curdlan-induced IL-10-producing CD4⁺ T cells and Foxp3⁺ Tregs, we next examined the expression levels of Foxp3 in CD4⁺ T cells stimulated in the presence of curdlan by intracellular staining and real-time PCR analysis. As shown in Fig. 3A, most IL-10-producing CD4⁺ T cells stimulated in the presence of curdlan did not express Foxp3, whereas a substantial proportion of CD4⁺ T cells stimulated in Treg conditions expressed Foxp3. Additionally, CD4⁺ T cells stimulated in the presence of curdlan did not express mRNA for Foxp3 (Fig. 3B). These results indicate that curdlan-induced IL-10-producing CD4⁺ T cells are distinct from Foxp3⁺ Tregs.

APCs and ICOS/ICOSL interaction are required for curdlan-induced development of IL-10-producing CD4⁺ T cells

It has been shown that not only myeloid cells such as DCs and macrophages but also some populations of T cells and B cells respond to curdlan (12, 35, 36). To address the cell type that primarily responds to curdlan during the induction of IL-10-producing CD4⁺ T cells, we next examined direct effects of curdlan on purified CD4⁺ T cells. In this experiment, purified CD4⁺ T cells from DO11.10 mice were stimulated with anti-CD3/CD28 Abs in the presence or absence of curdlan. As controls, purified CD4⁺ T cells from DO11.10 mice were stimulated with OVA peptide presented on purified splenic CD11c⁺ DCs in the presence or absence of curdlan. Consistent with data shown in Fig. 2B, curdlan induced IL-10 production in CD4⁺ T cells stimulated with OVA peptide presented on DCs (Fig. 4A, bottom panels). In contrast,

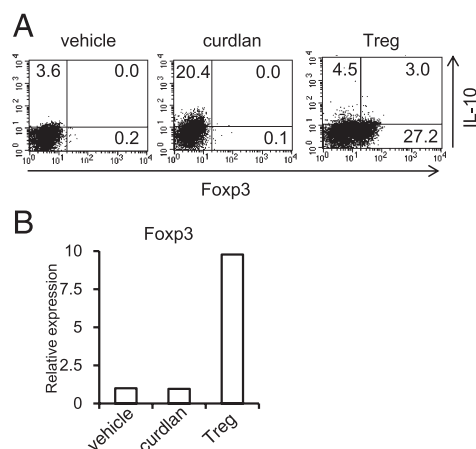


FIGURE 3. Curdlan-induced IL-10-producing CD4⁺ T cells are distinct from Foxp3⁺ Tregs. (A and B) Splenocytes from DO11.10 mice were stimulated with OVA peptide in the presence or absence of curdlan (100 μ g/ml) for 5 d. To induce Foxp3⁺ Tregs, CD4⁺ T cells from DO11.10 mice were stimulated under Treg conditions for 4 d. (A) After washing, cells were restimulated with PMA plus ionomycin and intracellular staining for IL-10 and Foxp3 in CD4⁺ T cells was performed. Results shown are representative of three independent experiments. (B) Live CD4⁺ T cells were collected and the levels of Foxp3 mRNA were evaluated by real-time PCR analysis. Shown are representative data of relative expression of Foxp3 to that in cells stimulated with vehicle ($n = 3$).

curdlan did not induce IL-10 production in CD4⁺ T cells stimulated with anti-CD3/CD28 Abs (Fig. 4A, upper panels). Taken together with a previous finding that surface expression of Dectin-1 is mainly detected on myeloid cells, including DCs (12), these results suggest that cells that primarily respond to curdlan are myeloid cells and that some cytokines derived from curdlan-stimulated myeloid cells and/or cell/cell interactions between myeloid cells and CD4⁺ T cells are involved in curdlan-induced IL-10 production in CD4⁺ T cells.

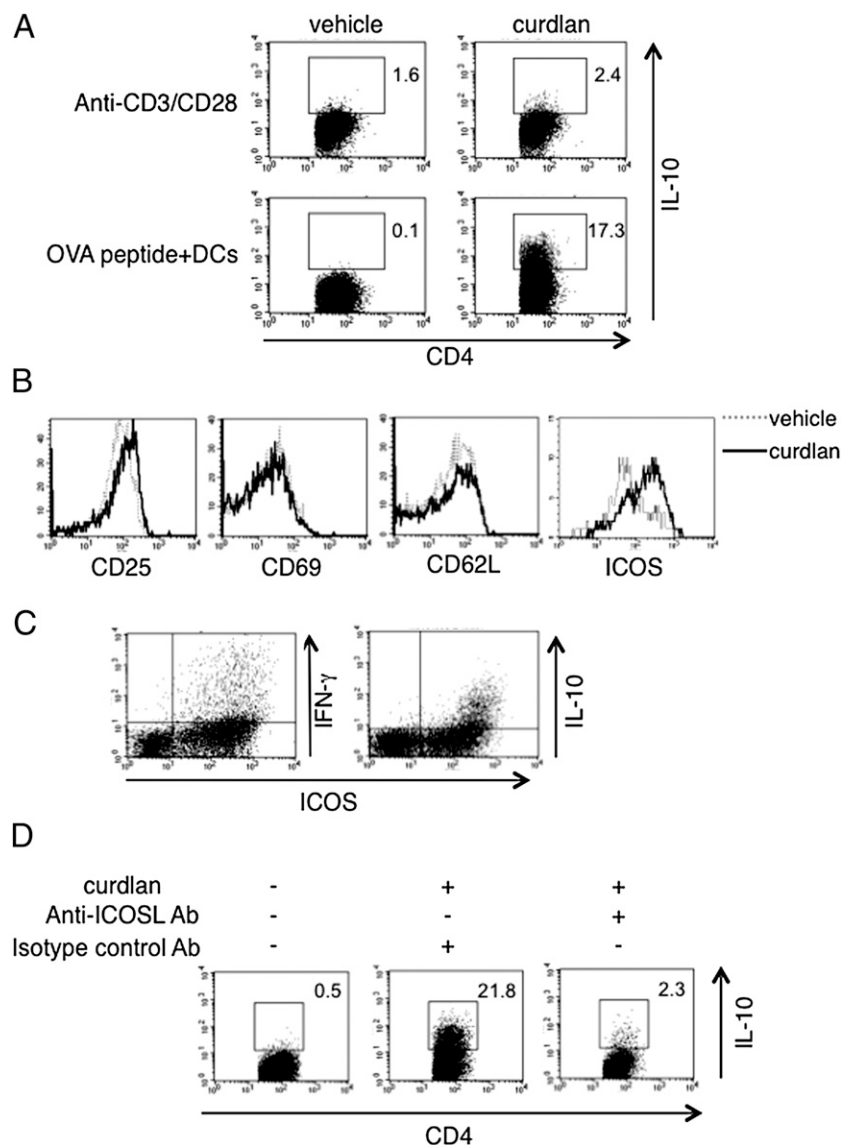
To further explore the mechanism by which curdlan induces IL-10 production in CD4⁺ T cells, we next examined the expression levels of surface molecules in CD4⁺ T cells stimulated in the presence or absence of curdlan. Whereas the levels of CD25, CD69, and CD62L in CD4⁺ T cells stimulated in the presence of curdlan were similar to those in CD4⁺ T cells stimulated in the absence of curdlan, the expression levels of ICOS were significantly increased in CD4⁺ T cells stimulated in the presence of curdlan (Fig. 4B). Moreover, FACS analysis of ICOS expression together with intracellular IL-10 or IFN- γ revealed that IL-10-producing CD4⁺ T cells expressed higher levels of ICOS than did IFN- γ -producing CD4⁺ T cells (Fig. 4C). Alternatively, the expression levels of ICOSL in curdlan-stimulated DCs were similar to those in control DCs (S. Kawashima, K. Hirose, and

H. Nakajima, unpublished observations). Importantly, blocking ICOS/ICOSL interaction by anti-ICOSL Ab inhibited curdlan-induced development of IL-10-producing CD4⁺ T cells (Fig. 4D). Taken together, these results suggest that ICOS/ICOSL interaction plays a critical role in curdlan-induced development of IL-10-producing CD4⁺ T cells.

STAT6 plays an essential role in curdlan-induced development of IL-10-producing CD4⁺ T cells

Given that T-bet and STAT6 have been suggested to be involved in the development of IL-10-producing CD4⁺ T cells in some situations (27, 37), we next examined the role of T-bet and STAT6 in curdlan-induced development of IL-10-producing CD4⁺ T cells. Splenocytes from DO11.10 mice, T-bet^{-/-} DO11.10 mice, and STAT6^{-/-} DO11.10 mice were stimulated with OVA peptide in the presence or absence of curdlan for 5 d and the development of IL-10-producing CD4⁺ T cells was examined. Importantly, the frequency of IL-10-producing CD4⁺ T cells was significantly decreased in STAT6^{-/-} DO11.10 mice as compared with that in DO11.10 mice ($n = 4$, $p < 0.005$) (Fig. 5A, 5B). The levels of IL-10 in the supernatants of CD4⁺ T cells stimulated in the presence of curdlan were also decreased in STAT6^{-/-} DO11.10 mice ($n = 3$, $p < 0.005$) (Fig. 5C). These results indicate that STAT6 is in-

FIGURE 4. Curdlan-induced development of IL-10-producing CD4⁺ T cells depends on ICOS/ICOSL interaction. **(A)** Purified CD4⁺ T cells from DO11.10 mice were stimulated with anti-CD3/CD28 Abs (upper panels) or OVA peptide presented on purified splenic CD11c⁺ DCs (lower panels) in the presence or absence of curdlan (100 μ g/ml) for 5 d. After washing, cells were restimulated with PMA plus ionomycin for 4 h and the intracellular staining for IL-10 was performed. Shown are representative FACS profiles gating on CD4⁺ T cells ($n = 3$). **(B)** Splenocytes from DO11.10 mice were stimulated with OVA peptide in the presence (solid lines) or absence (dotted lines) of curdlan for 5 d. The expression levels of CD25, CD69, CD62L, and ICOS on CD4⁺ T cells were analyzed by flow cytometry. Data are representative histograms of three independent experiments. **(C)** Splenocytes from DO11.10 mice were stimulated with OVA peptide in the presence or absence of curdlan for 5 d. After washing, cells were restimulated with PMA plus ionomycin for 4 h and surface expression of ICOS and intracellular IFN- γ or IL-10 was analyzed by flow cytometry. Representative FACS profiles of IFN- γ versus ICOS or IL-10 versus ICOS staining on CD4⁺ T cells are shown ($n = 4$). **(D)** Splenocytes from DO11.10 mice were stimulated with OVA peptide in the presence or absence of curdlan with anti-ICOSL Ab or isotype-matched control Ab for 5 d. After washing, cells were restimulated with PMA plus ionomycin and intracellular IL-10 staining was performed. Representative FACS profiles of IL-10 staining on CD4⁺ T cells are shown ($n = 4$).



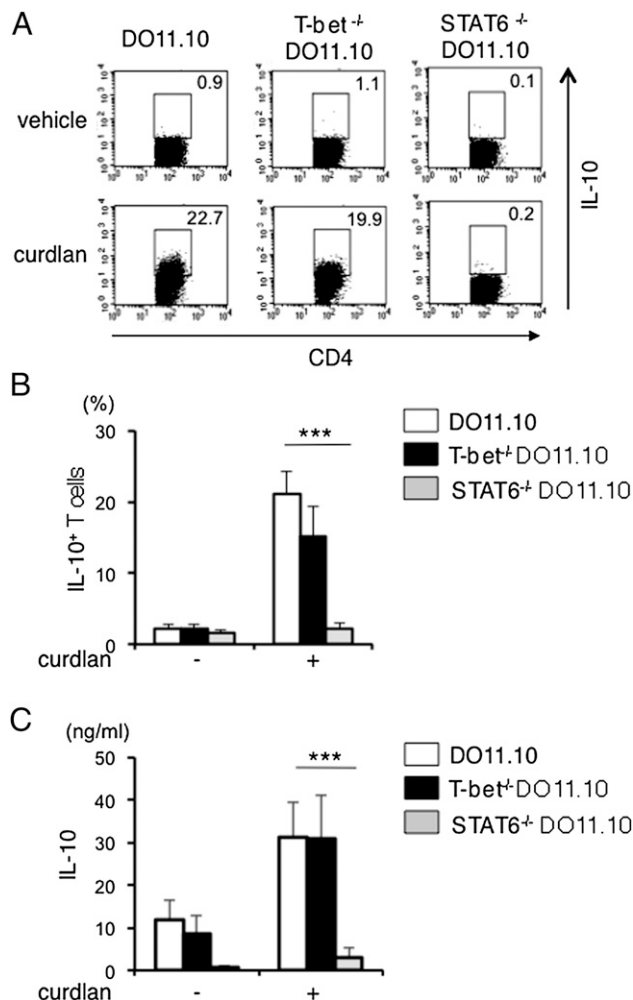


FIGURE 5. Curdlan-induced development of IL-10-producing CD4⁺ T cells depends on STAT6. (**A–C**) Splenocytes from DO11.10 mice, T-bet^{-/-} DO11.10 mice, and STAT6^{-/-} DO11.10 mice were stimulated with OVA peptide in the presence or absence of curdlan (100 µg/ml) for 5 d. After washing, cells were restimulated with PMA plus ionomycin for 4 h and the intracellular IL-10 staining was performed. (A) Representative FACS profiles of IL-10 staining on CD4⁺ T cells and (B) the frequency of IL-10⁺ cells in CD4⁺ T cells are shown ($n = 4$). (C) CD4⁺ T cells were isolated and restimulated with anti-CD3/CD28 Abs. The levels of IL-10 in the culture supernatants were measured by ELISA. Shown are means $\pm$ SD ($n = 3$). *** $p < 0.005$.

involved in the curdlan-induced development of IL-10-producing CD4⁺ T cells.

STAT6 expressed in CD4⁺ T cells is crucial for curdlan-induced development of IL-10-producing CD4⁺ T cells

Because STAT6 is expressed not only in CD4⁺ T cells but also in other populations of splenocytes, including DCs, we next examined cell types in which STAT6 expression is required for curdlan-induced development of IL-10-producing CD4⁺ T cells. CD4⁺ T cells were isolated from either DO11.10 mice or STAT6^{-/-} DO11.10 mice and then stimulated with OVA peptide presented on T cell-depleted splenocytes as APCs from either wild-type (WT) or STAT6^{-/-} mice in the presence or absence of curdlan. Whereas DO11.10 CD4⁺ T cells stimulated with T cell-depleted splenocytes from STAT6^{-/-} mice produced IL-10 in the presence of curdlan, STAT6^{-/-} DO11.10 CD4⁺ T cells stimulated with T cell-depleted splenocytes from WT mice failed to produce IL-

10 (Fig. 6A–C). These results suggest that STAT6 expressed in CD4⁺ T cells but not in APCs is required for curdlan-induced development of IL-10-producing CD4⁺ T cells.

CD4⁺ T cells stimulated in the presence of curdlan inhibit Ag-induced eosinophil recruitment into the airways

Finally, we examined the effect of adoptive transfer of CD4⁺ T cells stimulated in the presence of curdlan on Ag-induced airway inflammation. DO11.10 CD4⁺ T cells stimulated with OVA peptide in the presence or absence of curdlan were injected i.v. into OVA-sensitized BALB/c mice. Twenty-four hours later, mice were challenged with OVA inhalation and airway inflammation was evaluated at 48 h after OVA inhalation. As shown in Fig. 7A and 7B, the numbers of eosinophils as well as the levels of IL-5 and IL-13 in the BALF were decreased in mice injected with CD4⁺ T cells stimulated in the presence of curdlan as compared with those in mice injected with those stimulated in the absence of

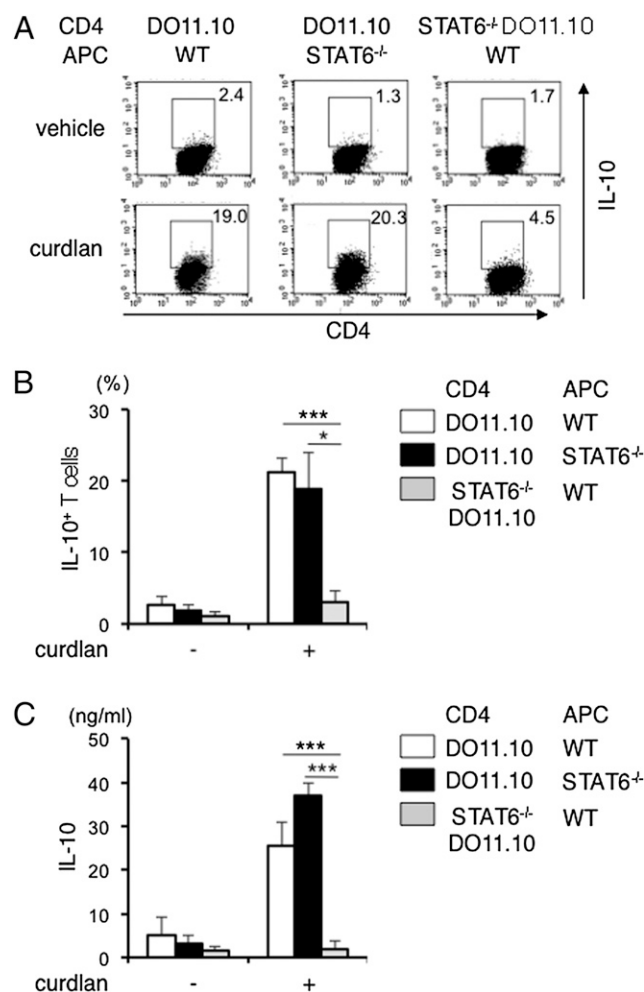


FIGURE 6. STAT6 expressed in CD4⁺ T cells is required for curdlan-induced development of IL-10-producing CD4⁺ T cells. (**A–C**) CD4⁺ T cells purified from DO11.10 or STAT6^{-/-} DO11.10 splenocytes were stimulated with OVA peptide presented on T cell-depleted splenocytes from WT or STAT6^{-/-} mice as APCs in the presence or absence of curdlan (100 µg/ml) for 5 d. After washing, cells were restimulated with PMA plus ionomycin for 4 h and intracellular IL-10 staining was performed. Representative IL-10 staining on CD4⁺ T cells (A) and the frequency of IL-10-producing cells in CD4⁺ T cells (B) are shown ($n = 4$). * $p < 0.05$, *** $p < 0.005$. (C) Live CD4⁺ T cells were restimulated with anti-CD3/CD28 Abs and the levels of IL-10 in the culture supernatants were measured by ELISA. Shown are means $\pm$ SD ($n = 3$). *** $p < 0.005$.

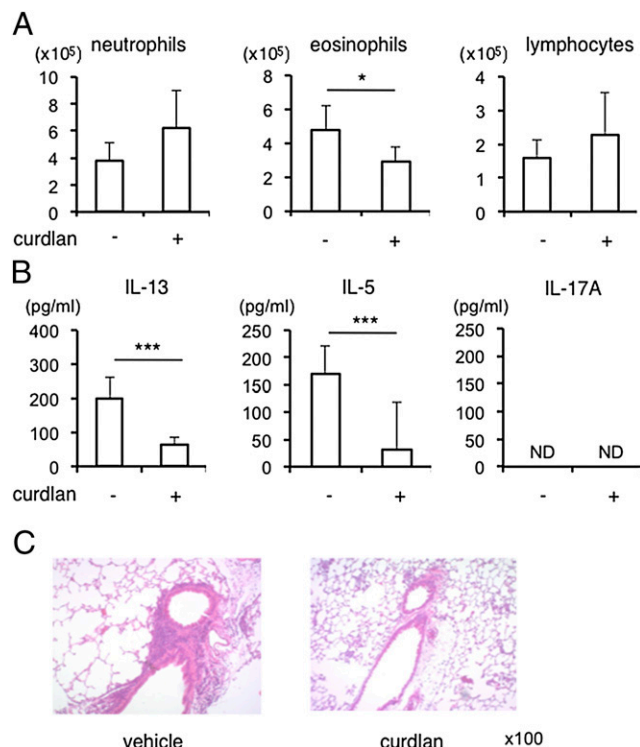


FIGURE 7. Ag-specific CD4⁺ T cells stimulated in the presence of curdlan inhibit Ag-induced eosinophil recruitment into the airways. (**A–C**) DO11.10 CD4⁺ T cells stimulated in the presence or absence of curdlan were injected to OVA-sensitized BALB/c mice. Twenty-four hours later, these mice were challenged with OVA inhalation. Forty-eight hours after OVA inhalation, the numbers of neutrophils, eosinophils, and lymphocytes in the BALF (**A**) and the levels of IL-5, IL-13, and IL-17A in the BALF (**B**) were evaluated. Data are means $\pm$ SD for five mice in each group. * $p < 0.05$, *** $p < 0.005$. (**C**) Histological analysis of the lungs was performed at 48 h after OVA inhalation (H&E staining). Shown are representative microphotographs of lung sections ($n = 5$). Original magnification $\times 100$.

curdlan ($n = 5$, $p < 0.005$). In contrast, the numbers of neutrophils in the BALF tended to be increased in mice injected with CD4⁺ T cells stimulated in the presence of curdlan (Fig. 7A). Histological analysis of the lung section revealed that peribronchiolar and perivascular inflammation was attenuated in mice injected with CD4⁺ T cells stimulated in the presence of curdlan (Fig. 7C). Taken together, these results suggest that curdlan-induced IL-10-producing CD4⁺ T cells exert potent inhibitory activities for allergic airway inflammation.

Discussion

In this study, we show that curdlan induces IL-10-producing CD4⁺ T cells in a STAT6-dependent manner and inhibits Ag-induced eosinophilic airway inflammation. We found that in vivo administration of curdlan inhibited Ag-induced eosinophil recruitment and Th2 cytokine production in the airways and induced IL-10-producing CD4⁺ T cells in the lung (Fig. 1). We also found that whereas curdlan did not inhibit Th2 cell differentiation in vitro (Fig. 2), curdlan induced IL-10-producing CD4⁺ T cells, which expressed c-Maf but not T-bet, GATA3, ROR γ t, or Foxp3, in a Dectin-1-dependent manner (Figs. 2, 3). Importantly, curdlan-induced development of IL-10-producing CD4⁺ T cells required the presence of APCs as well as ICOS/ICOSL interaction (Fig. 4). Curdlan-induced development of IL-10-producing CD4⁺ T cells also required intrinsic expression of STAT6 in CD4⁺ T cells (Figs. 5, 6). Finally, we showed that the adoptive transfer of CD4⁺

T cells, stimulated in the presence of curdlan, inhibited Ag-induced eosinophil recruitment into the airways (Fig. 7). Taken together with the fact that Dectin-1 is mainly expressed in myeloid cells, these results suggest that curdlan instructs APCs to induce the development of IL-10-producing CD4⁺ T cells that exhibit inhibitory functions for allergic inflammation.

We show that intrinsic expression of STAT6 in CD4⁺ T cells is indispensable for curdlan-induced development of IL-10-producing CD4⁺ T cells (Fig. 6). We also found that neutralizing anti-IL-4 Ab inhibited curdlan-induced development of IL-10-producing CD4⁺ T cells (S. Kawashima, K. Hirose, and H. Nakajima, unpublished observations). Despite the requirement of STAT6, we found that curdlan-induced IL-10-producing CD4⁺ T cells did not express IL-4 (Fig. 2B) or GATA3 (Fig. 2C), suggesting that curdlan-induced IL-10-producing CD4⁺ T cells are different from classical Th2 cells, although part of the mechanism for the development is shared with Th2 cells. These results also suggest that IL-4 produced by neighboring cells is involved in curdlan-induced STAT6-dependent development of IL-10-producing CD4⁺ T cells. Intriguingly, Mendel and Shevach (37) have reported that the development of CD4⁺ T cells that produce IL-10 but not IL-4 by exogenous IL-10 also depends on IL-4 and STAT6. These findings indicate that STAT6 plays indispensable roles not only for the differentiation of Th2 cells but also for the development of some kinds of IL-10-producing CD4⁺ T cells. Moreover, our findings suggest that STAT6-mediated GATA3 induction and subsequent Th2 cell differentiation might be suppressed by undefined mechanisms when CD4⁺ T cells are activated in the presence of curdlan-stimulated APCs.

We found that curdlan-induced IL-10-producing CD4⁺ T cells expressed high levels of c-Maf (Fig. 2C). Because c-Maf has been reported to activate the transcription of the IL-10 gene (32), the induction of c-Maf might be responsible for the production of IL-10 in CD4⁺ T cells stimulated in the presence of curdlan. However, whereas c-Maf has also been reported to activate the transcription of IL-4 (33) and IL-21 (38, 39), we found that curdlan-induced IL-10-producing CD4⁺ T cells did not produce IL-4 (Fig. 2B) or IL-21 (S. Kawashima, K. Hirose, and H. Nakajima, unpublished observations). The reason why curdlan-induced IL-10-producing CD4⁺ T cells do not produce IL-4 or IL-21 even though they express high levels of c-Maf is still unclear. Further studies are needed to evaluate the role of c-Maf in curdlan-induced development of IL-10-producing CD4⁺ T cells.

In addition to intrinsic factors of CD4⁺ T cells such as STAT6 and c-Maf, we showed that the activation of CD4⁺ T cells in the presence of APCs such as DCs was required for curdlan-induced development of IL-10-producing CD4⁺ T cells. Moreover, we demonstrated that ICOS was highly expressed in IL-10-producing CD4⁺ T cells (Fig. 4B, 4C) and that the blocking of ICOS/ICOSL interaction with anti-ICOSL Ab suppressed curdlan-induced development of IL-10-producing CD4⁺ T cells (Fig. 4D). Alternatively, we found that the expression levels of ICOSL in curdlan-stimulated DCs were similar to those in control DCs (S. Kawashima, K. Hirose, and H. Nakajima, unpublished observations). Because c-Maf is known to be a downstream molecule of ICOS signaling in some situations (38), it is plausible that stimulation of ICOS by ICOSL enhances the expression of c-Maf in CD4⁺ T cells and then leads to IL-10 production. Further studies are required to identify the factors that are derived from curdlan-stimulated DCs and induce ICOS upregulation in CD4⁺ T cells.

Recently, IL-27, a cytokine produced primarily by APCs after stimulation by microbial products or inflammatory mediators, has been shown to promote the generation of IL-10-producing Tr1 cells (40). IL-27 has also been shown to induce c-Maf, IL-21, IL-

21R, and ICOS expression in CD4⁺ T cells (31), raising the possibility that IL-27 is the DC-derived cytokine that induces IL-10 production and ICOS upregulation in CD4⁺ T cells in the presence of curdlan. However, in preliminary experiments, we found that curdlan-stimulated splenic CD11c⁺ DCs did not produce IL-27. Alternatively, we found that curdlan-stimulated splenic CD11c⁺ DCs produced IL-10 (S. Kawashima, K. Hirose, and H. Nakajima, unpublished observations), consistent with a previous finding that curdlan induces IL-10 production in bone marrow-derived DCs (41). Moreover, it has been shown that DCs exposed to IL-10 are a potent inducer of IL-10-producing T cells (42). Taken together, it is plausible that DC-derived IL-10, together with ICOS/ICOSL interaction, induces the development of IL-10-producing CD4⁺ T cells in the presence of curdlan.

Although several populations of IL-10-producing CD4⁺ T cells with inhibitory activities have been reported (43), lineage relationships among these populations are unclear owing to the lack of specific markers and a master transcription factor for each population of IL-10-producing CD4⁺ T cells. We found that curdlan-induced IL-10-producing CD4⁺ T cells lacked the expression of Foxp3 (Fig. 3), suggesting that this population was distinct from conventional Tregs. Further analyses are required to address the lineage relationships between curdlan-induced IL-10-producing CD4⁺ T cells and previously reported populations of IL-10-producing CD4⁺ T cells such as Tr1 cells, CD4⁺CD25⁺ LAP⁺ T cells, CD4⁺CD25⁺ LAG3⁺ T cells, and CD4⁺NKG2D⁺ T cells.

We showed that in vivo injection of curdlan attenuated Ag-induced eosinophil recruitment into the airways (Fig. 1A) and enhanced the accumulation of IL-10-producing CD4⁺ T cells in the lung (Fig. 1D, 1E). We also showed that the adoptive transfer of CD4⁺ T cells that were stimulated in the presence of curdlan inhibited Ag-induced eosinophil recruitment into the airways (Fig. 7). However, the inhibitory effect of adoptively transferred curdlan-stimulated CD4⁺ T cells was weaker than that of in vivo injection of curdlan. These results suggest that, in addition to the induction of IL-10-producing CD4⁺ T cells by curdlan, other mechanisms, such as the induction of regulatory macrophages by curdlan (44), might be involved in the curdlan-mediated inhibition of allergic airway inflammation in vivo.

In conclusion, we have demonstrated that a linear β -(1-3)-glucan curdlan inhibits allergic airway inflammation, in part through the induction of IL-10-producing CD4⁺ T cells. Although further studies are required for understanding the basis of the findings, our results should add a new insight into the therapeutic potential of β -glucan curdlan for allergic diseases, including asthma.

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Disclosures

The authors have no financial conflicts of interest.

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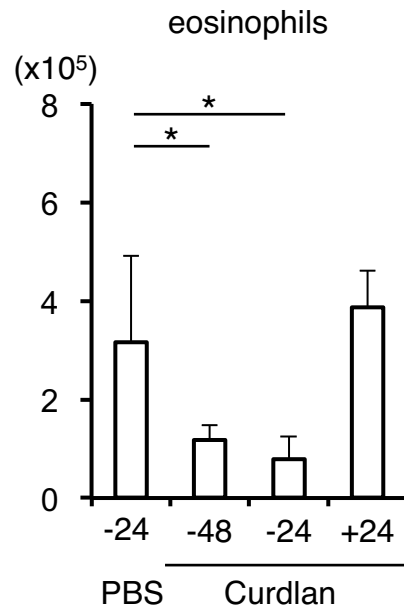


Figure S1. Curdlan inhibits antigen-induced eosinophil recruitment into the airways.

OVA-sensitized BALB/c mice were injected intraperitoneally with 3 mg of curdlan suspension or PBS at the indicated time period before (-) or after (+) the OVA inhalation. The numbers of eosinophils in the BALF were evaluated at 48 hours after OVA inhalation. Data are means $\pm$ SD for 4 mice in each group, * p <0.05.