activation through these receptors (29). Studies have implicated Notch in the activation (30–33) and differentiation (23, 25, 34) of cells of the peripheral immune system. RBP-J-deficient mice or mice expressing a dominant-negative form of the Mastermind-like protein suppressed both IL-4 production and Th2 responses (23, 35). We recently reported that Notch and Delta1 interactions in vivo inhibit the development of AHR and airway inflammation accompanied by heightened Th1 responses (36).

In this study, we define the important role of Notch-Notch ligand (Jagged1) interactions in vivo in the sensitization phase of the development of AHR and other lung allergic responses. *Jagged1* expression was enhanced on Ag-pulsed bone marrow-derived dendritic cells (BMDCs). Using an approach where Ag-pulsed BMDCs are transferred into naive recipients before allergen challenge, the full spectrum of lung allergic responses can be triggered. Using this model, we demonstrated that inhibition of Notch signaling on $CD4^+$ T cells using GSI or the inhibition of Jagged1 expression on BMDCs using small interfering RNA (siRNA) prevented the development of AHR and airway inflammation. In contrast, administration of Jagged1-Fc augmented AHR and airway inflammation. These results indicated that Notch-Notch ligand (Jagged1) interactions in vivo regulated the initiation of allergic airway disease by controlling the induction of IL-4 production, initiating Th2 differentiation.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 and IL-4-deficient ($IL\text{-}4^{-/-}$) mice were purchased from The Jackson Laboratory. The mice were housed under specific pathogen-free conditions and maintained on an OVA-free diet in the Biological Resources Center at National Jewish Health (Denver, CO). Both female and male mice, 8–12 wk of age, were used in these experiments and each experiment was independently performed at least three times with four mice per group ($n = 12$). Controls were matched to the deficient mice with regard to both age and gender in each experimental group. All experimental studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

BMDC generation

BMDCs were differentiated from bone marrow cells according to the procedure described by Inaba and colleagues (37, 38), with some modifications. Briefly, bone marrow cells were flushed from the femurs and tibias of C57BL/6 mice, washed, and cultured in complete medium (RPMI 1640 containing 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) from Invitrogen and recombinant mouse GM-CSF (10 ng/ml) and recombinant mouse IL-4 (10 ng/ml) from R&D Systems). Nonadherent granulocytes were removed after 48 h of culture and fresh complete medium was added every other day. All cultures were incubated at 37°C in 5% humidified CO_2. After 7 days of culture, > 80% of the cells expressed characteristic DC-specific markers ($CD11c^+$) as determined by flow cytometry. For some experiments, BMDCs on day 7 were cultured with OVA (200 μg/ml; Fisher Scientific) for 24 h. The LPS content in the solution was 1.6 ng/ml. These BMDCs were used in immunoblot analyses or total RNA was extracted from them for real-time PCR. All data were representative of at least three independent experiments.

For siRNA transfection, BMDCs were washed and plated in 24-well plates at a concentration of 2×10^5 cells/well in 400 μl of serum-free RPMI 1640. After 24 h of transfection, the cells were cultured with or without OVA for 24 h and washed three times with PBS.

BMDC transfection by siRNA

BMDCs were transfected with 21-bp siRNA sequences specific for Jagged1 (5′-CTCGTAATCCTTAATGGTT-3′) synthesized and annealed by the manufacturer (Dharmacon). Scrambled siRNA controls were used to establish a baseline response that could be compared with the levels in cells treated with target-specific siRNA. Transfection was conducted as described previously (39, 40). Briefly, 3 μl of 20 μM annealed siRNA was incubated with 3 μl of GenePorter (Gene Therapy Systems) in a volume of 94 μl of serum-free RPMI 1640 at room temperature for 30 min. This was then added to each well containing BMDCs and incubated for 4 h at 37°C. Three

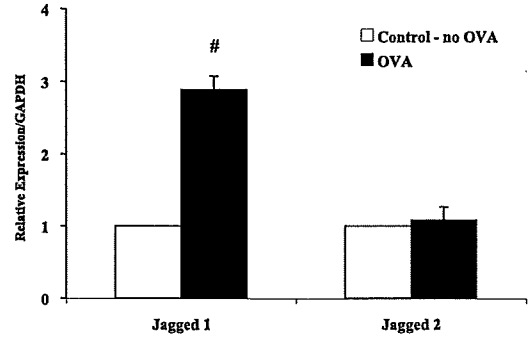

FIGURE 1. Real-time PCR analysis of Notch ligand expression in BMDCs pulsed with OVA. BMDCs from WT mice were incubated with or without OVA for 24 h and mRNA was isolated. The relative expression levels of Notch ligands (Jagged1 or Jagged2) were determined by quantitative real-time PCR. cDNA contents were normalized to levels of GAPDH. Results are from three independent experiments and the results for each group are expressed as mean ± SEM. #, $p < 0.05$, significant differences comparing BMDCs pulsed with OVA and BMDCs alone.

μl of GenePorter alone were used as mock controls. After incubation, 500 μl/well RPMI 1640 supplemented with 20% FCS was added to the cells. Twenty-four hours later, transfected or treated BMDCs were washed and used in subsequent experiments.

$CD4^+$ T cell preparation

Purification of $CD4^+$ T cells from C57BL/6 mice was conducted as previously described (41). Briefly, spleen cells from naive mice were harvested by mincing the tissues and subsequently passing them through a stainless steel sieve. After washing with PBS, mononuclear cells were isolated by Histopaque gradient centrifugation (Sigma-Aldrich). Purification of $CD4^+$ T cells was conducted by negative selection using a mouse $CD4^+$ T cell recovery column kit (purity, >95%; Cedarlane Laboratories) in accordance with the manufacturer's instructions. Purity of $CD4^+$ T cell populations after purification exceeded 95% as assessed by flow cytometry. For some experiments, isolated $CD4^+$ T cells were cultured with GSI (20 μM) (dibenzazepine; Calbiochem-EMD Biosciences) or DMSO (0.1% final concentration) for 24 h.

Preparation of soluble Jagged1-Fc

The extracellular portion of Jagged1 cDNA (the sequence between nt 1 and 3276) was originally obtained by PCR using C57BL/6 splenocytes as a template. A cDNA for the Fc portion of human IgG1 (IgG1-Fc) was constructed in-frame to the 3′ end of the cDNAs encoding the extracellular region of Jagged1 in the expression vector pcDNA3.1 (Invitrogen). Chinese hamster ovary cells were transfected with these pcDNA3.1 plasmid-containing cDNAs for the Jagged1-Fc protein using the FuGENE 6 transfection reagent (Roche Applied Science). After culture of these cells for several days, the supernatants were collected and soluble protein was purified from the concentrated supernatant using HiTrap protein G HP (Amersham Biosciences) according to the manufacturer's instructions.

In vitro coculture of $CD4^+$ T cells with BMDCs and Jagged1-Fc

Isolated $CD4^+$ T cells from WT mice were pretreated with GSI (GSI/CD4) or DMSO (DMSO/CD4) and IL-2 for 24 h. GSI/CD4 or DMSO/CD4 were cocultured with BMDCs previously pulsed with OVA (200 μg/ml) at a ratio of 10:1 for 5 days. After culture, viable cells were restimulated with plate-bound anti-CD3 plus anti-CD28 (2 μg/ml each; R&D Systems) for 3 days. All BMDCs were treated with mitomycin C (50 mg/ml; Sigma-Aldrich) before being cultured with $CD4^+$ T cells. Culture supernatants were harvested for cytokine analysis. In some in vitro experiments, isolated $CD4^+$ T cells (2×10^6) from WT mice were stimulated with plate-bound anti-CD3 together with plate-bound Jagged1-Fc (5 μg/ml) or human IgG (5 μg/ml) for 5 days. Viable $CD4^+$ cells were then restimulated with plate-bound anti-CD3 and anti-CD28 (2 μg/ml, respectively) for 2 days. Supernatants were collected and evaluated by ELISA. All data are representative of at least three independent experiments conducted in triplicate.

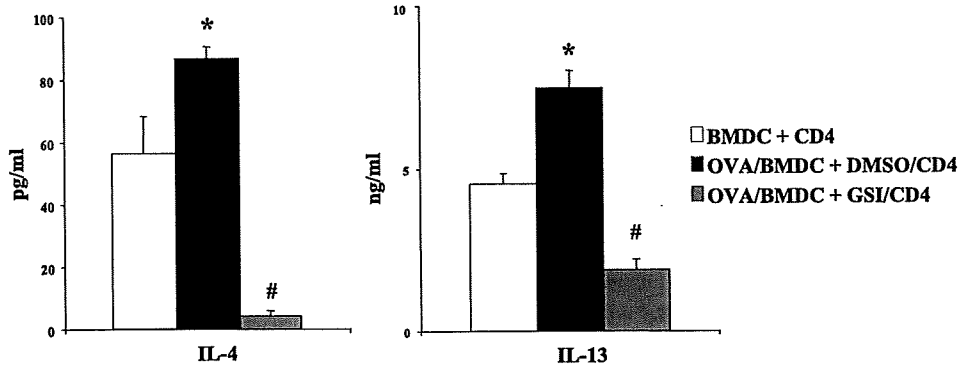

FIGURE 2. Cytokine production from $CD4^+$ T cells pretreated with GSI and cocultured with OVA-pulsed BMDCs. Isolated naive $CD4^+$ T cells from WT mice were incubated with GSI (GSI/CD4) or DMSO (DMSO/CD4) and IL-2 for 24 h. Subsequently, GSI/CD4 or DMSO/CD4 T cells were cocultured with OVA/BMDCs. After 5 days, viable $CD4^+$ T cells were restimulated with plate-bound anti-CD3 and anti-CD28 (2 μg/ml, respectively) for 3 days. Supernatants were collected and evaluated by ELISA. The results are representative of three independent experiments conducted in triplicate and are expressed as means ± SEM. #, $p < 0.05$, significant differences comparing OVA/BMDC plus GSI/CD4, BMDC plus CD4 and OVA/BMDC plus DMSO/CD4; and *, $p < 0.05$, significant differences comparing OVA/BMDC plus DMSO/CD4 and BMDC plus CD4.

Adoptive transfer of BMDCs and $CD4^+$ T cells and administration of Jagged1-Fc

In these transfer protocols, BMDCs were cultured with OVA (200 μg/ml) 24 h and instilled intratracheally (2×10^6 cells/recipient). Ten days after the transfer of BMDCs, mice were challenged via the airways with OVA (1% in saline solution) for 20 min on three consecutive days. Forty-eight hours after the last allergen challenge, all assays were conducted. For adoptive transfer of T cells, naive $CD4^+$ T cells(5×10^6) pretreated with GSI (GSI/CD4) or DMSO (DMSO/CD4) were administered i.v. through the tail vein to IL-$4^{-/-}$ recipients, followed by intratracheal administration of OVA-pulsed BMDCs. IL-$4^{-/-}$ mice that received no cells served as controls.

In the Jagged1-Fc protocol, soluble Jagged1-Fc was injected i.p. at a daily dose of 200 μg beginning 4 days before through the day following transfer of OVA-pulsed BMDCs in WT mice before challenge with OVA. As a control, human IgG (200 μg) was administered in the same manner.

To assess the effect of Jagged1 knockdown on BMDC activity in vivo, BMDCs transfected with siRNA-Jagged1 were instilled intratracheally into naive C57BL/6 or IL-$4^{-/-}$ mice that received naive $CD4^+$ T cells (5×10^6). The WT or IL-$4^{-/-}$ recipients of untreated BMDCs, BMDCs transfected with reagent alone (mock-treated), or control siRNA (siRNA-scrambled) served as controls.

Assessment of airway function

Airway function was assessed as previously described by measuring changes in lung resistance (R_L) in response to increasing doses of inhaled methacholine (MCh) (42). Data are expressed as percentage change from baseline R_L values obtained after inhalation of saline. The baseline R_L

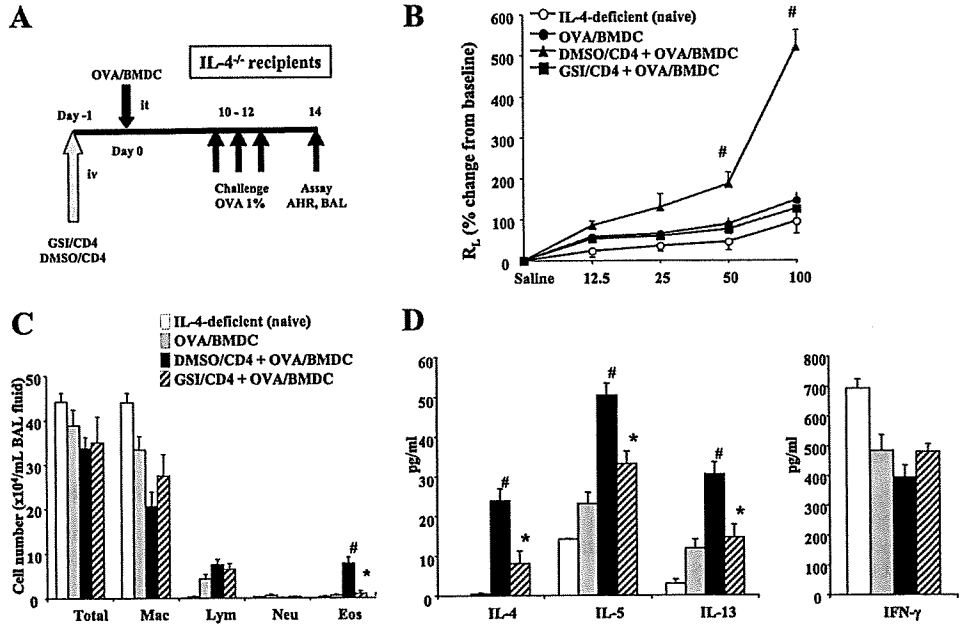

FIGURE 3. Transfer of GSI/$CD4^+$ T cells and OVA/BMDCs fails to restore lung allergic responses in IL-$4^{-/-}$ mice. *A*, Experimental protocol. Naive WT $CD4^+$ T cells (5×10^6) pretreated with GSI (GSI/CD4) or DMSO (DMSO/CD4) were administered i.v. into IL-$4^{-/-}$ mice, followed by intratracheal administration of OVA/BMDCs (2×10^6). Ten days after the cell transfers, mice received three OVA challenges. IL-$4^{-/-}$ mice that received no $CD4^+$ T cells or OVA/BMDCs alone are also shown. *B*, R_L values were obtained in response to increasing concentrations of inhaled MCh. *C*, Cellular composition of BAL fluid. *D*, Cytokine levels in BAL fluid. Total, Total cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils. Data represent the means ± SEM ($n = 12$ in each group). #, $p < 0.05$, significant differences comparing DMSO/CD4 plus OVA/BMDC recipients and GSI/CD4 plus OVA/BMDC recipients, OVA/BMDC recipients, or IL-$4^{-/-}$ mice (naive); *, $p < 0.05$, significant differences comparing GSI/CD4 plus OVA/BMDC recipients and DMSO/CD4 plus OVA/BMDC recipients.

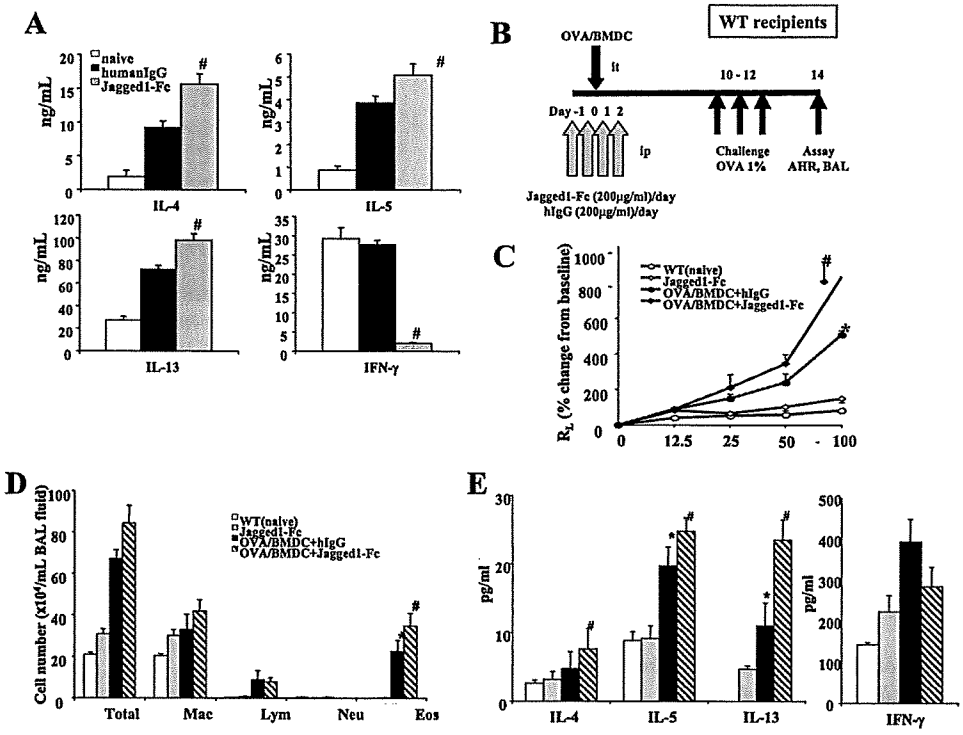

FIGURE 4. Jagged1-Fc stimulated IL-4 production and enhanced allergen-induced AHR and airway inflammation. *A*, Isolated $CD4^+$ T cells from naive WT mice were stimulated with platebound anti-CD3/anti-CD28 and Jagged1-Fc or human IgG (5 μg/ml). Supernatants were collected and cytokine production evaluated. Data represent the mean ± SEM from three independent experiments conducted in triplicate. #, $p < 0.05$, significant differences between naive $CD4^+$ T cells or $CD4^+$ T cells stimulated with anti-CD3/anti-CD28 and human IgG and $CD4^+$ T cells stimulated with anti-CD3/anti-CD28 and Jagged1-Fc. *B*, Experimental protocol. *C*, AHR. *D*, Cell composition in BAL fluid. *E*, BAL cytokine levels. WT mice were treated with Jagged1-Fc or human IgG following transfer of OVA-pulsed BMDCs and before OVA challenges. Mice received OVA challenges following transfer of Jagged1-Fc alone (Jagged1), OVA-pulsed BMDCs with human IgG (OVA/BMDC+hIgG), and OVA-pulsed BMDCs with Jagged1-Fc (OVA/BMDC+Jagged1). Naive WT mice that received neither Jageed1-Fc nor human IgG were also shown. The results for each group are expressed as the mean ± SEM ($n = 12$ in each group). #, $p < 0.05$, significant differences between OVA/BMDC plus Jagged1 recipients, OVA/BMDC plus human IgG recipients, Jagged1 recipients, or WT mice (naive); *, $p < 0.05$, significant differences between OVA/BMDC plus human IgG recipients and Jagged1 recipients or WT mice (naive). Total, Total cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

responses to saline in the individual groups were not significantly different from each other.

Bronchoalveolar lavage (BAL)

Immediately following measurement of AHR, lungs were lavaged with HBSS (1 × 1 ml at 37°C) and total leukocyte numbers were analyzed. Differential cell counts were performed under light microscopy by counting at least 200 cells on cytocentrifuged preparations (Shandon Cytospin 2; Thermo Scientific), stained with Leukostat (Fisher Diagnostics), and differentiated by standard hematological procedures in a blinded fashion.

Preparation of RNA and real-time PCR

Total RNA was extracted from BMDCs or siRNA-treated BMDCs using an RNeasy mini kit (Qiagen). Two micrograms of total RNA was used in each reaction primed with oligo-dT to obtain c-DNA. Then, 3 μl of the synthesized cDNA was used as the template for real-time PCR. Real-time cDNA primers and probes for Jagged1 targeted by siRNA were as follows: forward primer, 5′-CAAAAACCCCATCGAGAAACA-3′; reverse primer, 5′-TCCTGATTTTTGACATTTTCGAGTT-3′; probe, 5′-ACGGTCCCCA TTAAGGATTACGAGAA-3′. Jagged2 and GAPDH primers and probes were obtained from Applied Biosystems. The real-time PCRs were performed on an ABI 7700 sequence detection system (Applied Biosystems) with cycling parameters of 50°C for 2 min, 95°C for 10 min, and 40 repeats at 95°C for 15 s and 60°C for 1 min. The ΔΔ cycle threshold method was performed for relative quantification of mRNA expression.

Western blot analysis

Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were treated as recommended by the Ab manufacturer for Jagged1 (Novus Biologicals). For detection of the specific protein, a sensitive chemiluminescence method was used with an appropriate IgG Ab linked to ab HRP Ab (Pierce).

Measurement of cytokines

Cytokine levels in the BAL fluid and cell culture supernatants were measured by ELISA as previously described (43). IL-4, IL-5, IFN-γ (BD Pharmingen), and IL-13 (R&D Systems) ELISAs were performed according to the manufacturers' directions. The lower limits of detection were 4 pg/ml for IL-4, IL-5, and IL-13 and 10 pg/ml for IFN-γ.

Statistical analysis

Results were expressed as the mean ± SEM. The *t* test was used to determine differences between two groups and the Tukey-Kramer test was used for comparisons between multiple groups. Measured values may not be normally distributed because of the small sample sizes. Nonparametric analysis using the Mann-Whitney *U* test or Kruskal-Wallis test was also used to confirm that the statistical differences remained significant even if the underlying distribution was uncertain. The *p* values for significance were set to 0.05 for all tests.

Results

Expression of Notch ligand on OVA-pulsed BMDCs

Because expression of the Notch ligands Jagged1 and Jagged2 on APCs has been associated with the development of Th2 responses in vitro (22), we first analyzed the levels of their expressions in BMDCs cultured with/without OVA for 24 h using real-time PCR. The expression of Jagged1 was significantly higher in BMDCs cultured with OVA compared with that in BMDCs cultured in

medium alone (Fig. 1). However, the expression of Jagged2 in BMDCs cultured with OVA showed little increase over that seen with BMDCs alone.

Notch signaling controls cytokine production from CD4$^+$ T Cells cocultured with OVA-pulsed BMDCs

To assess whether Notch signaling of CD4$^+$ T cells in vitro affected Th1/Th2 polarization, we analyzed cytokine production in cocultures of OVA-pulsed BMDCs (OVA/BMDC) with naive CD4$^+$ T cells that were or were not pretreated with GSI to prevent Notch signaling. We previously showed that GSI pretreatment markedly inhibited Notch signaling (36). Naive CD4$^+$ T cells isolated from spleens of WT mice were incubated with DMSO (DMSO/CD4) or GSI (GSI/CD4) for 24 h in the presence of IL-2 (20 U/ml). DMSO/CD4 or GSI/CD4 were cocultured with OVA/BMDC, followed by restimulation with plate-bound anti-CD3 and anti-CD28 for 24 h. Supernatants from GSI/CD4 cocultured with OVA/BMDC contained significantly lower levels of IL-4 and IL-13 compared with cultures containing DMSO/CD4 (Fig. 2). These data indicated that pharmacologic inhibition of Notch signaling resulted in markedly reduced cytokine production from CD4$^+$ T cells.

Inhibition of Notch signaling on CD4$^+$ T cells decreases their ability to promote allergen-induced AHR and airway inflammation in IL-4$^{-/-}$ recipients

To test the functional consequences of GSI treatment of CD4$^+$ T cells in vivo, we used a BMDC transfer model in which CD4$^+$ T cells were shown to be essential for the development of AHR and airway inflammation (44). To isolate directly the function of transferred CD4$^+$ T cells and not that of host CD4$^+$ T cells in the initiation of Th2-type allergic airway inflammation, IL-4$^{-/-}$ recipients were used. GSI/CD4 or DMSO/CD4 were transferred into IL-4$^{-/-}$ mice before OVA/BMDC administration, followed by three OVA challenges (Fig. 3*A*). As shown in Fig. 3*B*, IL-4$^{-/-}$ mice were incapable of developing AHR or eosinophilic airway inflammation despite receiving OVA/BMDC before challenge. However, IL-4$^{-/-}$ mice that received both OVA/BMDC and DMSO/CD4 developed increased AHR as illustrated by significant increases in R_L in response to increasing doses of inhaled MCh (Fig. 3*B*). In parallel to the increases in airway responsiveness, the inflammatory cell composition of BAL fluid was altered with significant increases in eosinophil numbers (Fig. 3*C*). In contrast, IL-4$^{-/-}$ recipients of OVA/BMDC and GSI/CD4 did not develop an increase in airway reactivity above that seen in naive IL-4$^{-/-}$ mice or IL-4$^{-/-}$ recipients of OVA/BMDC alone. Recipients of GSI/CD4 T cells also did not show increases in BAL eosinophil numbers.

The balance between levels of Th1 and Th2 cytokines has been proposed to play an important role in the development of allergic airway inflammation (45). IL-4, IL-5, and IL-13 levels in the BAL of IL-4$^{-/-}$ recipients of DMSO/CD4 T cells were increased, whereas GSI/CD4 T cell recipients showed smaller increases in IL-4, IL-5, and IL-13, but no differences in IFN-γ levels (Fig. 3*D*).

Effect of Jagged1-Fc on the response of CD4$^+$ T cells in WT mice

To define the effects of Jagged1-Fc on cytokine production directly, isolated CD4$^+$ T cells (2×10^6) from WT mice were stimulated with plate-bound anti-CD3 alone or together with plate-bound Jagged1-Fc or human IgG (5 μg/ml) for 5 days and then restimulated with plate-bound anti-CD3 and anti-CD28 (2 μg/ml, respectively) for an additional 2 days. Supernatants were collected and evaluated by ELISA. As shown in Fig. 4*A*, when T cells were initially cultured with anti-CD3 and human IgG and then restimulated with anti-CD3/anti-CD28, levels of IL-4, IL-5, and IL-13 increased over those seen in the absence of stimulation. Following the addition of Jagged1 in the initial phase, the levels of these cytokines were further increased. In parallel, the levels of IFN-γ were decreased and the decreases were augmented by Jagged1.

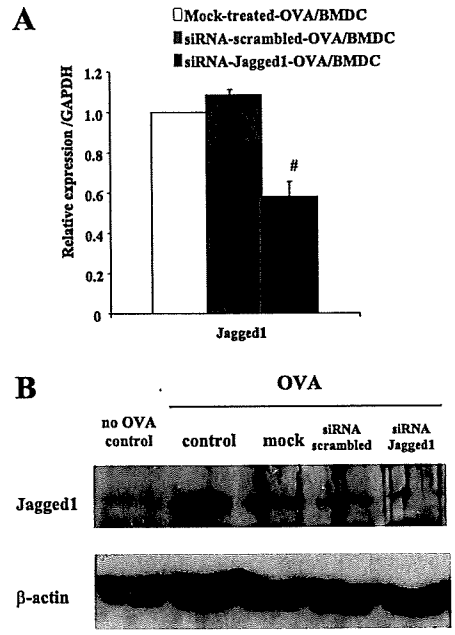

FIGURE 5. Gene silencing in OVA/BMDCs using Jagged1-specific siRNA. BMDCs were transfected with siRNA-Jagged1, siRNA-scrambled, reagent (Gene Porter) alone (mock-treated), or non-transfected cells (control). Transfected BMDCs was incubated with OVA for 24 h and RNA from these transfected BMDCs was collected to assess the expression of Jagged1 by real-time PCR and by Western blotting. *A*, Real-time PCR analysis of Jagged1 levels in transfected BMDCs. Mock-treated results were taken as 1. Results are from three independent experiments. The data for each group are expressed as means ± SEM. #, $p < 0.05$, significant differences between siRNA-Jagged1 and mock-treated or siRNA-scrambled BMDCs. *B*, Jagged1 protein levels in transfected BMDCs. BMDCs were unmanipulated (control), transfected with reagent alone (mock), siRNA scrambled, or siRNA-Jagged1. Transfected BMDCs were incubated with OVA for 24 h and cell lysates from these transfected BMDCs were collected to assess the expression of Jagged1 by Western blotting. β-Actin was used as a loading control. Representative of one of three similar experiments.

To directly determine whether the administration of Jagged1 in vivo regulates AHR and airway inflammation, WT mice were treated with Jagged1-Fc or human IgG as a control following the transfer of OVA-pulsed BMDCs and before OVA challenge (Fig. 4*B*). The administration of Jagged1-Fc markedly enhanced AHR compared with the administration of (control) human IgG following the transfer of OVA-pulsed BMDCs and OVA challenge (Fig. 4*C*). In parallel, the administration of Jagged1-Fc to WT mice increased the numbers of eosinophils and the levels of Th2 cytokines in the BAL compared with controls (Fig. 4, *D* and *E*). These data indicated that administration of the Notch ligand Jagged1 can further enhance the development of lung allergic responses, even in WT mice.

Gene silencing in BMDCs treated with Jagged1 siRNA

To further analyze the importance of Notch-Jagged1 interactions, we used the ability of siRNA to reduce *Jagged1*-specific gene

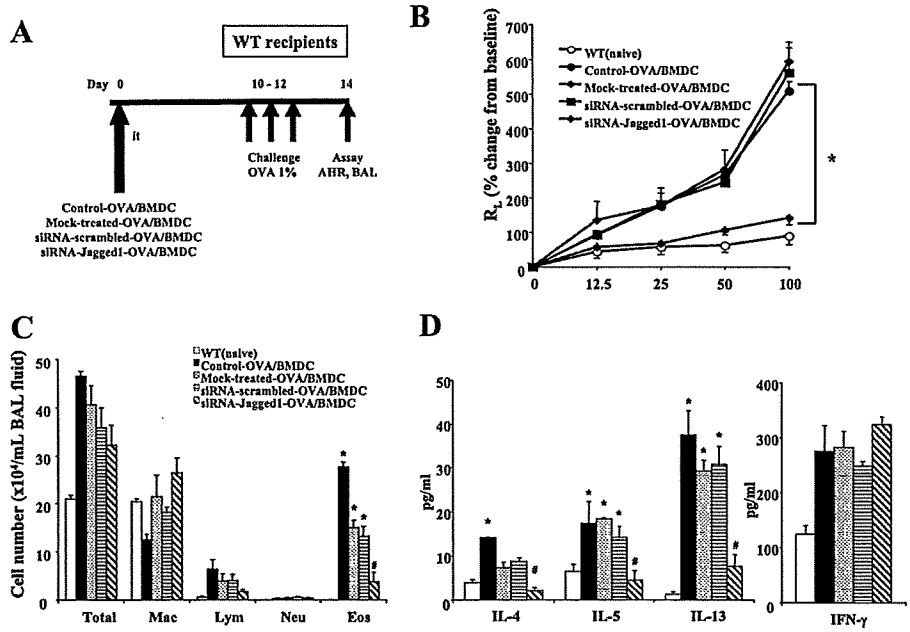

FIGURE 6. Administration of OVA/BMDCs transfected with siRNA-Jagged1 fails to restore lung allergic responses in WT recipients. *A*, Experimental protocol. BMDCs were transfected with siRNA-Jagged1, siRNA scrambled, or mock treated. Subsequently, transfected and untreated BMDCs were incubated with OVA for 24 h before transfer into WT mice. Ten days later, mice received three daily OVA challenges. *B*, AHR. R_L values were obtained in response to increasing concentrations of inhaled MCh. *, $p < 0.05$, significant differences. *C*, Cellular composition of BAL fluid. *D*, Cytokine levels in BAL fluid. Data represent the means ± SEM ($n = 12$ in each group). #, $p < 0.05$, significant differences between siRNA-Jagged1-OVA/BMDC recipients vs OVA/BMDC, mock-treated-OVA/BMDC, and siRNA-scrambled-OVA/BMDC recipients; *, $p < 0.05$, significant differences between OVA/BMDC, mock-treated-OVA/BMDC, or siRNA-scrambled-OVA/BMDC recipients and WT mice (naive). Total, Total cells; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

expression in BMDCs cultured with OVA. The expression of *Jagged1* mRNA and protein levels in BMDCs were analyzed by real-time PCR using primers flanking the siRNA target sequence and by Western blotting, respectively. The levels of Jagged1 mRNA in BMDCs transfected with siRNA-Jagged1 were decreased by ~50% compared with levels in BMDCs transfected with siRNA-scrambled or mock-treated BMDCs (Fig. 5*A*). Following pulsing with OVA, Jagged1 protein levels in BMDCs were markedly increased compared with those of nonpulsed BMDCs (Fig. 5*B*). Following transfection of OVA-pulsed BMDC with siRNA-Jagged1, Jagged1 protein levels were markedly reduced.

Inhibition of Jagged1 in BMDCs decreases their ability to induce allergen-dependent AHR and airway inflammation in WT mice

To determine the functional consequences of *Jagged1* gene silencing in OVA/BMDC, we monitored the effects of the transfer of gene-silenced OVA/BMDC into naive WT mice before allergen challenge (Fig. 6*A*). This DC-dependent protocol has been shown to be dependent on the Ag pulsing of DCs before transfer and allergen challenge in naive WT mice (44). WT mice received either OVA/BMDC, siRNA-Jagged1-OVA/BMDC, mock-treated-OVA/BMDC, or siRNA-scrambled-OVA/BMDC intratracheally before the three daily OVA challenges. WT recipients of OVA/BMDC, mock-treated OVA/BMDC, or siRNA-scrambled-OVA/BMDC developed significant increases in MCh-induced AHR and airway eosinophilia (Figs. 6, *B* and *C*). This was in contrast to the responses following transfer of siRNA-Jagged1-OVA/BMDC, where AHR and airway eosinophilia failed to develop. The levels of Th2 cytokines in the BAL fluid paralleled the findings for AHR and airway eosinophilia with no significant increases in Th2 cytokine levels in the BAL fluid of recipients of the Jagged1-silenced BMDC (Fig. 6*D*). There were no significant differences among the recipients of any of the OVA/BMDC groups when levels of IFN-γ were examined (Fig. 6*D*).

Inhibition of Jagged1 on BMDCs decreases allergen-induced $CD4^+$ T Cell/IL-4-dependent AHR and airway inflammation in $IL\text{-}4^{-/-}$ recipients

To complement the findings in WT mice and to confirm the direct impact of transferred $CD4^+IL\text{-}4^+$ T cells on the initiation of lung allergic responses in vivo, these same populations of OVA/BMDCs were transferred into $IL\text{-}4^{-/-}$ recipients before the transfer of naive (WT) $CD4^+$ T cells and OVA challenge (Fig. 7*A*). AHR to inhaled MCh was significantly increased in $IL\text{-}4^{-/-}$ recipients of OVA/BMDCs and naive $CD4^+$ T cells, but not in those receiving either alone (Fig. 7*B*). Transfer of mock-treated-OVA/BMDCs or siRNA-scrambled-OVA/BMDCs together with $CD4^+$ T cells before OVA challenge resulted in similar increases in MCh-induced AHR, whereas transfer of siRNA-Jagged1-OVA/BMDCs failed to increase AHR. In parallel to the assessment of lung function, the inflammatory cell composition of BAL fluid was also different, with recipients of siRNA-Jagged1-OVA/BMDCs failing to develop significant BAL eosinophilia (Fig. 7*C*). In addition, Th2 cytokine levels in the BAL fluid of $IL\text{-}4^{-/-}$ recipients of siRNA-Jagged1-OVA/BMDCs did not demonstrate the increases seen in the other recipient groups (Fig. 7*D*); levels of IFN-γ were similar in all groups. The transfer of $CD4^+IL\text{-}4^-$ T cells alone was incapable of restoring any of the responses. These data defined the requirement for Jagged1-expressing DCs and $CD4^+IL\text{-}4^+$ T cells in the initiation of lung allergic responses in vivo.

Discussion

In allergic asthma, accumulation of $CD4^+$ T cells producing Th2 cytokines has been commonly observed in BAL fluid and lung biopsies (4, 45, 46). There is also abundant evidence from animal

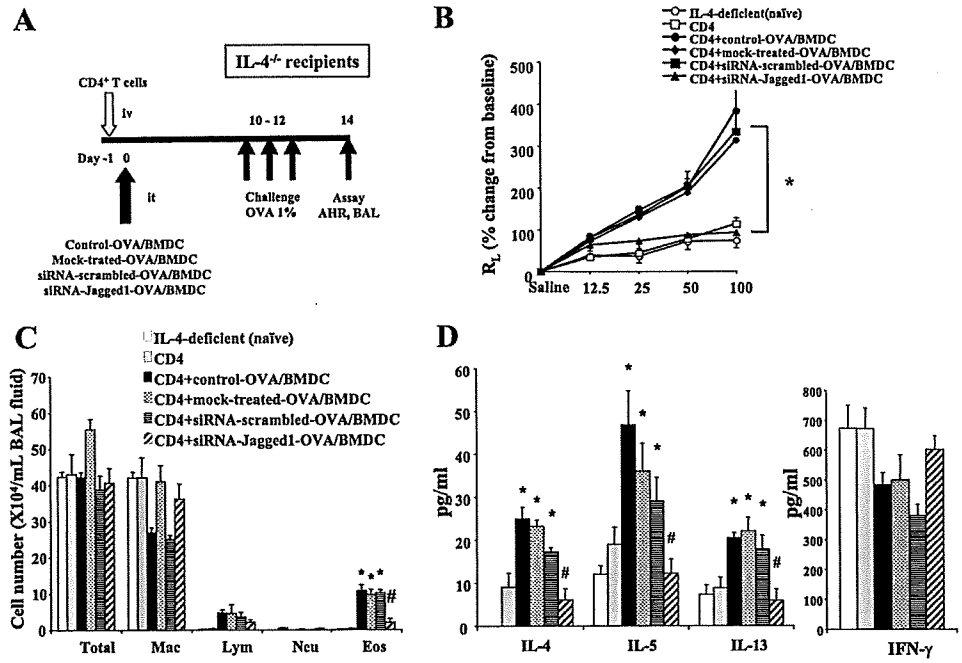

FIGURE 7. Allergen-induced AHR and airway inflammation are not restored in IL-4$^{-/-}$ recipients of siRNA-Jagged1-transfected BMDCs and CD4$^+$ T cells. *A*, Experimental protocol. IL-4$^{-/-}$ mice received naive CD4$^+$ T cells i.v. and unmanipulated or transfected BMDCs, including siRNA-Jagged1-BMDCs, siRNA-scrambled-BMDCs, or mock-treated-BMDCs, following 24 h incubation with OVA. Ten days later, mice received three daily OVA challenges. Data represent the means ± SEM ($n = 12$ in each group). *B*, AHR. R_L values were obtained in response to increasing concentrations of inhaled MCh. *, $p < 0.05$, significant differences. *C*, Cellular composition of BAL fluid. #, $p < 0.05$, significant differences comparing CD4 plus siRNA-Jagged1-OVA/BMDC recipients and CD4 plus OVA/BMDC, CD4 plus mock-treated-OVA/BMDC, or CD4 plus siRNA-scrambled-OVA/BMDC recipients. *D*, Cytokine levels in BAL fluid. #, $p < 0.05$, significant differences comparing CD4 plus siRNA-Jagged1-OVA/BMDC recipients and CD4 plus OVA/BMDC, CD4 plus mock-treated-OVA/BMDC, and CD4 plus siRNA-scrambled-OVA/BMDC recipients; *, $p < 0.05$, significant differences between CD4 plus OVA/BMDC, CD4 plus mock-treated-OVA/BMDC, or CD4 plus siRNA-scrambled-OVA/BMDC recipients and IL-4$^{-/-}$ mice (naive) or CD4 recipients.

studies that IL-4 plays a major role in the development of AHR and the influx of eosinophils as a result of Th2 cell differentiation (10, 18, 37). Thus, IL-4 is an effective and essential initiator of Th2 differentiation, and the development of effective Th2 responses in vivo and in vitro depends on IL-4 (47–50). However, the events that trigger IL-4 production, beginning with the initial encounters of naive T cells with APCs, remain less well understood, especially in vivo and in the development of allergic asthma.

In the present study, Jagged1 but not Jagged2 was found to be up-regulated in BMDCs pulsed with OVA compared with non-pulsed BMDCs, and the elevation was maintained for at least 24 h. The increases in gene expression were paralleled by increases in Jagged1 protein levels. Several reports have suggested that Notch ligand signaling by activated DCs is involved in directing specific Th1 and Th2 polarization on Notch-expressing T cells (22, 23, 25, 51). In vitro, Jagged-expressing APC cell lines were shown to induce Th2 cytokine production preferentially, whereas Delta-expressing cell lines induced IFN-γ production (22). As a rule, the Notch ligands tend to be expressed in a more highly restricted pattern than their receptors. Recent reports have noted the up-regulation of Jagged2 expression on DCs by helminths with induction of Th2 differentiation (52). Cholera toxin treatment also stimulated Jagged2 expression in a c-kit-dependent manner in BMDCs from WT mice that was linked to the development of allergic airway inflammation (53). In experimental autoimmune encephalomyelitis, anti-Jagged1 Ab exacerbated whereas anti-Delta1 Ab reduced the severity of clinical disease (54). Investigation of these Notch pathways has underscored the complex role of APCs in the initiation and regulation of Th1/Th2 differentiation. Notch signaling has been shown to direct Th2 differentiation via GATA3 (55, 56) through IL-4 receptor signaling in a STAT6-dependent fashion (14, 47, 57). However, little is known about the interaction between Notch on CD4$^+$ T cells and Notch ligand on APCs pulsed with OVA in the development of lung allergic responses.

To investigate the role of Notch-Jagged1 interactions on CD4$^+$ T cells and APCs in the context of allergen-driven responses, several in vitro and in vivo approaches were followed, including the inhibition of Notch signaling in CD4$^+$ T cells using a GSI, the silencing of *Jagged1* expression in APCs, and the use of a BMDC transfer protocol. This BMDC transfer model was shown to induce AHR and airway eosinophilia, but to a less robust extent than in models where mice were systemically sensitized and then challenged via the airways (19). Nonetheless, these responses following BMDC transfer were shown to be dependent on both IL-4-producing CD4$^+$ T cells and APCs pulsed with Ag (19). To first determine the consequences of inhibiting Notch signaling in CD4$^+$ T cells on the development of lung allergic responses, we used the BMDC transfer model in which the transfer of allergen-pulsed BMDCs intratracheally before allergen challenge was shown to be essential (44). To focus on the role of Notch in inducing IL-4 from CD4$^+$ T cells in this model, we used IL-4-deficient recipient mice that have been shown to exhibit a significantly reduced ability to develop AHR and airway eosinophilia, accompanied by decreased BAL IL-13 levels (18, 58). In these IL-4$^{-/-}$ recipients, reconstitution of the full development of lung allergic responses could be achieved by adoptive transfer of naive (WT) CD4$^+$ T cells, followed by the transfer of OVA-pulsed BMDCs and allergen challenge. In contrast, the transfer of GSI-treated CD4$^+$ T cells failed to restore AHR, eosinophilic inflammation, or Th2 cytokine levels in these IL-4-deficient recipients. These data indicate that in the context of APC interactions with CD4$^+$ T cells in vitro or in vivo, Notch signaling is a critical step for the differentiation of naive

$CD4^+$ T cells to a Th2 (IL-4 producing) phenotype and the development of lung allergic responses.

Given this role for Notch signaling in the $CD4^+$ T cells, we next determined whether the increase in *Jagged1* gene expression and protein levels in OVA-pulsed BMDCs was the critical ligand-mediated event in these responses. A number of approaches were used to confirm the importance of this ligand in triggering Th2 differentiation and the development of lung allergic responses. When Jagged1-Fc was administered in vivo to WT mice together with OVA-BMDCs followed by OVA airway challenges, Jagged1 increased all lung allergic responses, including AHR, airway eosinophilia, and BAL Th2 cytokines levels. In complementary studies, the transfection of BMDCs to silence *Jagged1* gene expression was conducted. Transfection of BMDCs with siRNA was effective in silencing targeted genes and provided a means to examine the capacity of allergen-pulsed and siRNA-modified BMDCs to alter the allergen-specific immune response and Th polarization in recipients (39). In OVA-pulsed BMDCs, we demonstrated that siRNA could be used to target the expression of Jagged1 at the transcription and protein levels, resulting in functional consequences. In WT mice, the transfer of BMDCs silenced by siRNA-Jagged1 failed to trigger AHR or airway (eosinophilic) inflammation. As a result of the silencing of *Jagged1* by siRNA in OVA/BMDC, Th2 cytokine production in BAL fluid was inhibited without affecting IFN-γ production. Together, these data demonstrate the importance of Notch signaling in $CD4^+$ T cells and *Jagged1* expression in APCs for the development of these responses in the WT recipients.

Th2 polarization has been reported to occur through both IL-4-dependent and -independent pathways (47–50). To directly examine the impact of Notch-Jagged1 interactions in the context of initiating IL-4 production, we examined the outcomes of administering OVA/BMDCs silenced by siRNA-Jagged1 to IL-4-deficient mice that received naive (WT) $CD4^+$ T cells. The transfer of siRNA-Jagged1 OVA/BMDCs together with $CD4^+$ T cells failed to restore AHR, airway eosinophilia, or BAL Th2 cytokine levels in these recipients. Thus, it appears that the blockade of Jagged1 on DCs did not influence or default to the expression of other Notch ligands (e.g., Delta4), enhancing IFN-γ production.

These studies demonstrate for the first time that the effects of either preventing Notch signaling by GSI treatment of $CD4^+$ T cells or Notch ligand expression through siRNA-Jagged1 silencing of BMDCs resulted in attenuation of the full array of lung allergic responses. Central to this failure was the inability of the $CD4^+$ T cells to undergo Th2 differentiation, produce IL-4, and initiate the lung allergic cascade. These data identify the Notch-Jagged1 pathway as a critical initiator and regulator of the development of allergen-induced, Th2-mediated AHR and lung allergic inflammation. Manipulation of this pathway may be particularly effective in the treatment of allergic airway disease.

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Disclosures

The authors have no financial conflict of interest.

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Protein–tyrosine phosphatase-kappa regulates CD4$^+$ T cell development through ERK1/2-mediated signaling

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ABSTRACT

T cells express diverse antigen-specific receptors and are required for eradicating pathogens and transformed cells. T cells expressing CD4 acquire helper effector functions and those expressing CD8 exert cytotoxic activity after antigen recognition. The protein–tyrosine phosphatase, receptor type kappa (PTPRK) is mutated in LEC rats, resulting in impaired CD4$^+$ T cell development in the thymus. However, the molecular mechanism of PTPRK controlling CD4$^+$ T cell development remains unclear. We demonstrate herein that inhibition of PTPRK by transducing a dominant negative form of the intracellular domain of PTPRK (PTPRK-ICD-DN) in bone marrow-derived stem cells suppresses the development of CD4$^+$ T cells. The inhibition of PTPRK by PTPRK-ICD-DN or short-hairpin RNA for PTPRK attenuates ERK1/2 phosphorylation in T cells after PMA and ionomycin stimulation. Total thymocytes from LEC rats also showed weaker phosphorylation of ERK1/2 after PMA and ionomycin stimulation than control thymocytes. Furthermore, inhibition of PTPRK by PTPRK-ICD-DN suppressed MEK1/2 and c-Raf phosphorylation, which is required for ERK1/2 phosphorylation. These data indicate that PPTRK positively regulates ERK1/2 phosphorylation, which impacts CD4$^+$ T cell development.

Introduction

Reversible protein phosphorylation is one of the major systems that controls cell functions and protein–tyrosine phosphatases (PTPs) have major roles in reversible protein phosphorylation. PTPs have been classified into cytoplasmic and receptor types (PTPRs) which are further categorized into eight subfamilies [1]. Human receptor-type protein–tyrosine phosphatase PTPRK belongs to the MAM-subfamily and possesses two cytoplasmatic PTP domains and an intracellular juxtamembrane region [1]. The precise physiological role of PTPRK as well as that of many other PTPRs is still unclear. Nevertheless, the ability of PTPRK and of other PTPRs to mediate homophilic or heterophilic interactions among cells [2], together with the observation that their expression is up-regulated by cell density [3], which strongly suggest a crucial role of these proteins in modulating signals induced by cell–cell contact.

The LEC rat is a model animal for Wilson's disease caused by a mutation in the copper transporting ATPase gene [4]. Agui et al. originally identified that LEC rats have impaired CD4$^+$ T cell development in the thymus while mature CD4$^+$ T cells gradually accumulate in the peripheral lymphoid organs with age [5]. We and another group demonstrated by linkage analysis that LEC rats have a large deletion in PTPRK that is responsible for defective CD4$^+$ T cell development [6,7]. However, the precise mechanism of how mutation in PTPRK affects CD4$^+$ T cell development remains unclear.

We demonstrate herein that inhibition of PTPRK suppresses ERK1/2 phosphorylation in a T cell hybridoma and total thymocytes of LEC rats stimulated by PMA and ionomycin. These data suggest that PTPRK controls CD4$^+$ T cell development at least partly through ERK1/2-mediated signaling.

Materials and methods

Animals. Mice and LEC rats were maintained in the Animal Research Center of The University of Tokushima under specific pathogen-free conditions and all animal work was approved by the animal research committee of The University of Tokushima.

Reagents and antibodies. Antibodies to MAP kinase (p44/42 MAP Kinase), phosphorylated MAP kinase (phospho-p44/42 MAP Kinase (Thr202/Tyr204)), MEK1/2, phosphorylated MEK1/2 (phospho-MEK1/2 (Ser217/221)), c-Raf, and phosphorylated c-Raf (phospho-c-Raf (Ser338)) were purchased from Cell Signaling Technology, Inc. (Boston, MA).

Plasmid construction. The full-length murine PTPRK gene was amplified using fwd: 5′-AACTTCTCCCAAACTCGCCATG-3′ and rev: 5′-GCAAATAGTCTCAGCGAAC-3′ primers, and cloned into pMX-IG

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retroviral vector. PTPRK-ICD fragment was cloned into a retroviral vector pMX-IG following PCR-based amplification with the fwd: 5′-ATGGGGAACACACGTCAG-3′ and rev: 5′-GCAAATAGTCTCAGC GAAC-3′ primers. Site directed mutagenesis was performed in the first catalytic domain to change cysteine to serine by PCR to make PTPRK-ICD-DN with the fwd: 5′-GGTGCTGGGCGCACAGGCTGT-3′ and rev: 5′- AGCACTGGAGTGTACGACAAT-3′ primers. The plasmid vectors of miR-Luc and miR-PTPi were prepared according to the protocol as described [6].

Cell culture, transfection, and infection. DO11.10 T cell hybridoma cells and Jurkat cells were maintained in RPMI1640 (Dako) medium supplemented with 10% fetal calf serum (Gibco), 2 mM L-glutamine, penicillin, and streptomycin. The retroviral vector constructs were transfected into the retrovirus packaging cell line Plat-E [8] with Gene Juice (Novagen, USA), according to the manufacturer's instructions. Forty-eight hours after transfection culture supernatants were collected and used for infection of DO11.10 or Jurkat cells with 8 μg/ml Polybrene (Chemicon International, CA, USA) by centrifugation at 2600 rpm for 90 min.

Flow cytometry. Total spleen cells were stained with PE-conjugated anti-mouse CD4 (eBioscience) and FITC-conjugated anti-mouse CD8 (eBioscience) antibodies. Cells were stained with anti-CD4 and anti-CD8 antibodies for 15 min at 4 °C and washed with FACS buffer (2% FBS, 0.05% NaN_2). Flow cytometry was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA). Thymocytes were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 mAbs. After staining, cells were sorted by using a cell sorter (JSAN, Bay Bioscience, Japan).

Semi-quantitative RT-PCR. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The isolated RNA was converted into cDNAs using Oligo (dT) primers (Invitrogen) and an Omniscript RT Kit (Qiagen). Semi-quantitative RT-PCR was performed for 38 cycles using a PCR Thermal Cycler (TaKaRa). Primer sequences were as follows: PTPRK full-length fwd: 5′-TATAGGCACTGAGGTGCA-3′ and rev: 5′-CTGCTGGCTCAA CAGA-3′. The PTPRK gene expression levels were normalized by the corresponding gene expression levels of mouse β-actin.

Western blotting. The cells were harvested and lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, protease inhibitor cocktail (Roche, Germany), phosphatase inhibitor cocktail 2 (Sigma, St. Louis, USA)). The cell lysates were resolved on SDS–PAGE and transferred to PVDF membranes (Atto, Japan). The membrane was then immunoblotted with each antibody after blocking. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore, Bedford, MA).

Generation of bone marrow reconstituted mice. Lineage marker-negative bone marrow cells (Lin-cells) were isolated with paramagnetic beads (Miltenyi Biotech). The Lin-cells were infected with retroviruses of pMX-IG and PTPRK-ICD-DN and transferred into C57BL/6 mice previously irradiated with 9.5 Gy using an irradiator (Hitachi Koki, Japan). Seven to eight weeks after transplantation, mice were sacrificed and single cell suspensions were prepared from spleen and thymus and analyzed by flow cytometry.

Results

PTPRK gene expression was detected in every thymocyte subset

As PTPRK is required for $CD4^+$ T cell development [6,7], we examined the expression of PTPRK in thymocyte subsets of C57BL/6 mice using semi-quantitative PCR. A comparable level of PTPRK gene expression was detected among double-negative (DN), double-positive (DP), and CD4 and CD8 single-positive (SP) cells (Fig. 1A). We also checked expression of PTPRK in the DO11.10 T cell hybridoma by semi-quantitative PCR and found high expression of PTPRK (data not shown). These data suggest that regulation of PTPRK function or expression of a PTPRK sub-

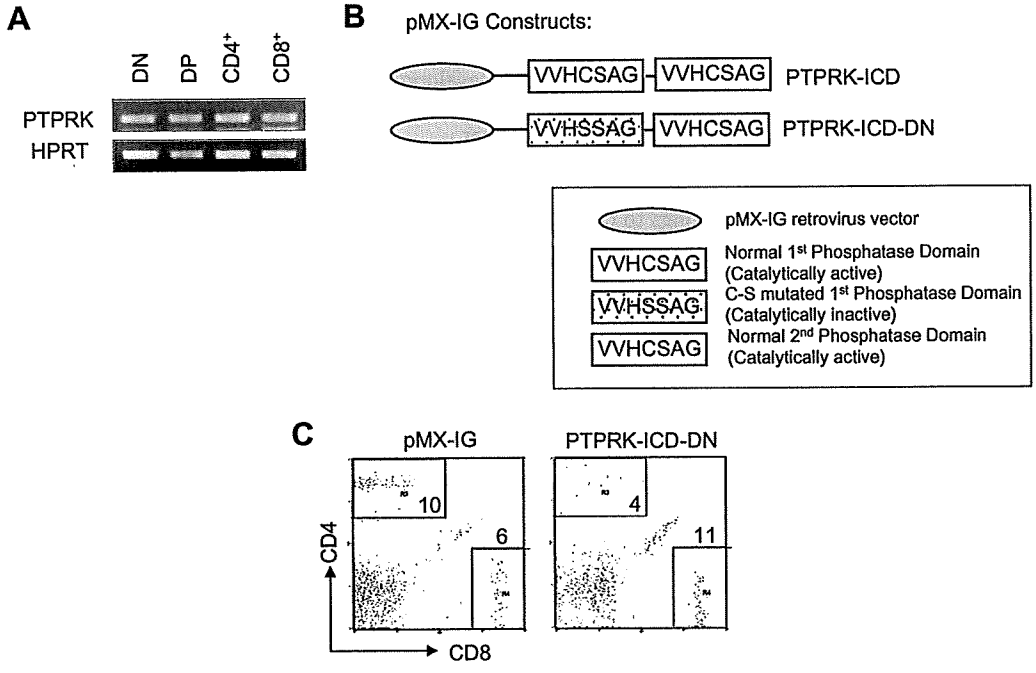

Fig. 1. PTPRK-ICD-DN impairs CD4 single-positive T cell development. Double-negative ($CD4^-CD8^-$), double-positive ($CD4^+CD8^+$) and single-positive $CD4^+$ or $CD8^+$ T cells were isolated from the thymus of C57BL/6 mice by cell sorting. (A) Expression of PTPRK in each population was examined by semi-quantitative PCR. The results were obtained from at least three independent experiments. (B) Schematic diagram of the PTPRK-ICD and the PTPRK-ICD-DN constructs used in this study. (C) The Lin-cells were infected with pMX-IG and PTPRK-ICD-DN and then cells were transferred to previously irradiated C57BL/6 mice. Spleen cells seven weeks after transfer were stained by anti-CD4 and CD8 mAb and the expression of both molecules was analyzed by flow cytometry. The results were obtained from at least three independent experiments.

strate but not PTPRK expression itself is involved in selective loss of $CD4^+$ T cells.

Construction and expression of PTPRK-ICD-DN

In order to make dominant negative form of PTPRK (PTPRK-ICD-DN), a point mutation was introduced at nucleotide 4335 in the first catalytic domain (ICD) of the gene, which led to a cysteine to serine substitution in the corresponding protein (Fig. 1B). The dominant negative mutation in the first intercellular phosphatase domain abolishes enzymatic activity and suppresses endogenous protein-tyrosine phosphatase function [9]. To confirm that loss of function of the PTPRK is responsible for $CD4^+$ T cell development, we generated bone marrow chimera mice with PTPRK-ICD-DN-transduced BM cells. Lineage marker-negative bone marrow cells were isolated with paramagnetic beads and infected with retroviruses of control pMX-IG or PTPRK-ICD-DN. Then each population was transferred into C57BL/6 mice previously irradiated with 9.5 Gy. Seven to eight weeks after transplantation, T cell development was evaluated by flow cytometry. We found that the development of $CD4^+$ SP cells was impaired in PTPRK-ICD-DN transduced cells compared with control cells (Fig. 1C). These data indicate that PTPRK-ICD-DN is able to exert selective suppressive function during $CD4^+$ T cell development.

PTPRK regulates Erk1/2 phosphorylation

A number of studies have demonstrated that ERK1/2 activity is crucial for CD4 and CD8 T cell maturation [10–12]. Therefore, we tested if PTPRK is involved in the ERK1/2 signaling pathway.

We first stimulated total thymocytes from LEC rats with PMA and ionomycin and evaluated Erk1/2 phosphorylation (Fig. 2A). We found less phosphorylation of ERK1/2 in LEC rat thymocytes compared with those from control rats (Fig 2A), suggesting that PTPRK might affect the ERK signaling pathway.

We then transduced the pMX-IG, PTPRK-ICD and PTPRK-ICD-DN constructs into the DO11.10 (mouse) or Jurkat (human) T cell lines. Seventy-two hours after transduction, the cells were stimulated with PMA and ionomycin (0, 5, 15, and 30 min). We found decreased phosphorylation of ERK1/2 in PTPRK-ICD-DN transduced cells in both cell lines, while such a change was not observed in PTPRK-ICD transduced cells (Fig. 2B). We also tested the effect of PTPRK on the phosphorylation of ERK1/2 in DO11.10 cells transduced with miR-Luc and miR-PTPi and observed that phosphorylated ERK1/2 was less in miR-PTPi than control miR-Luc transduced cells (Fig. 2C). These data indicate that ERK1/2 phosphorylation is impaired by suppressing PTPRK functions.

PTPRK regulates MEK1/2 phosphorylation

Since ERK1/2 is phosphorylated by MEK1/2, we examined the phosphorylation of MEK1/2 in pMX-IG, PTPRK-ICD or PTPRK-ICD-DN transduced DO11.10 cells after PMA and ionomycin stimulation. We found decreased phosphorylation of MEK1/2 in the PTPRK-ICD-DN-transduced cells compared with that in PTPRK-ICD-transduced cells (Fig. 3A). We also checked the phosphorylation of MEK1/2 in DO11.10 cells with miR-PTPi, and found decreased phosphorylation of MEK1/2 in miR-PTPi-transduced cells compared to miR-Luc-transduced cells (Fig. 3B). c-Raf is MAP3 kinase and most frequently phosphorylates MEK1/2. Therefore, we analyzed the influence of

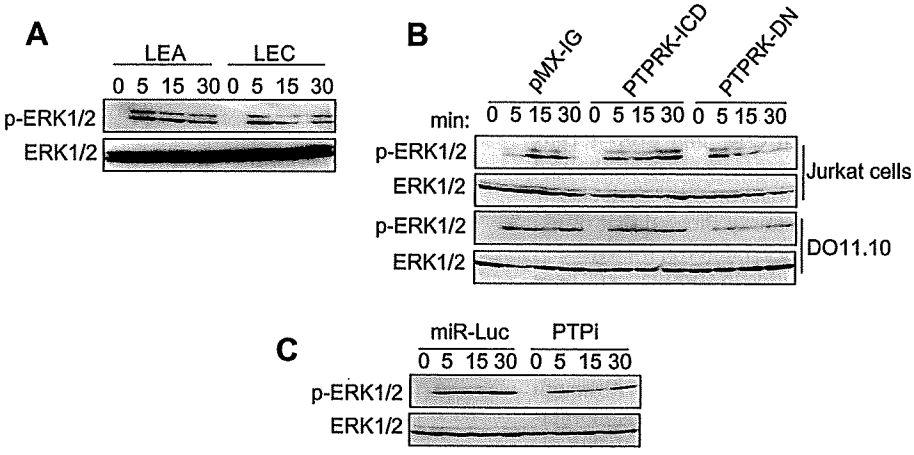

Fig. 2. Decreased phosphorylation of ERK1/2 in the DO11.10 and Jurkat T cell lines. The p-ERK1/2 phosphorylation was measured by Western blot analysis after cells were treated with PMA (25 ng/μl) and ionomycin (1 ng/μl) for the indicated times (0, 5, 15 or 30 min). (A) Total thymocytes of LEA and LEC rats, (B) pMX-IG, PTPRK-ICD, and PTPRK-ICD-DN transduced DO11.10 and Jurkat T cell lines, and (C) miR-Luc or miR-PTPi transduced DO11.10 T cell line. The results were obtained from at least three independent experiments.

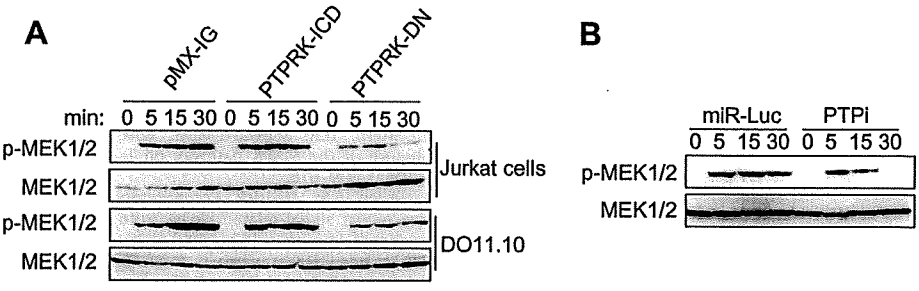

Fig. 3. PTPRK-ICD-DN inhibits MEK phosphorylation. Western blot analysis of p-MEK1/2 expression from transduced pMX-IG, PTPRK-ICD, and PTPRK-ICD-DN constructs into the DO11.10 and Jurkat T cell lines (A), and transduced miR-luc or miR-PTPi constructs into DO11.10 T cells (B). Western blot analysis was performed as in this figure. The results were obtained from at least three independent experiments.

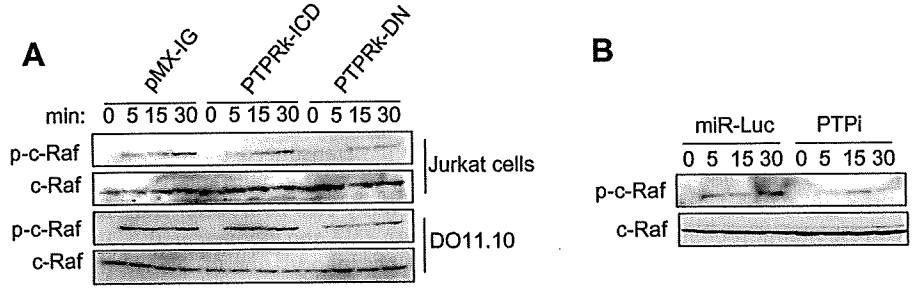

Fig. 4. PTPRK affects c-Raf/MEK/ERK pathway. Western blot analysis of p-cRaf expression from transduced pMX-IG, PTPRK-ICD, and PTPRK-ICD-DN constructs into the DO11.10 and Jurkat T cell lines (A), and transduced miR-luc or miR-PTPi constructs into DO11.10 T cells (B). Western blot analysis was performed as in Fig. 3. The results were obtained from at least three independent experiments.

PTPRK on the phosphorylation of c-Raf. Phosphorylation of c-Raf was impaired in both the cells with PTPRK-ICD-DN (Fig. 4A) or miR-PTPi (Fig. 4B), compared with each control. These data demonstrate that PTPRK affects the c-Raf/MEK/ERK pathway.

Discussion

The LEC rat has a large deletion in the PTPRK gene that causes the selective impairment of $CD4^+$ T cell development in the thymus [6,7]. However, the molecular mechanisms by which deletion of PTPRK affects $CD4^+$ T cell development remain unclear. We demonstrated herein that inhibition of PTPRK suppresses MAPK activation in T cells. Since previous research provided evidence that the ERK1/2 pathway is important for $CD4^+$ T cell development, our data suggest that low MAPK activation due to inhibition of PTPRK is involved in the decreased $CD4^+$ T cell development observed in LEC rats.

A previous report demonstrated that the thymocyte-specific expression of a hypersensitive ERK2 transgene favored CD4 development, and likewise MEK inhibitors favored CD8 development [10]. Subsequent studies have revealed that mice lacking ERK1/2 have impaired positive selection of both $CD4^+$ and $CD8^+$ T cells [12]. Further analysis using T cell receptor transgenic mice showed that deficiency in ERK1/2 has a greater impact on $CD4^+$ than $CD8^+$ T cell lineage progression [12]. Our present study showed that transduction of PTPRK-ICD-DN in T cells decreased ERK1/2, MEK1/2 and c-Raf phosphorylation after PMA and ionomycin stimulation. These data indicate that inhibition of PTPRK downregulates the ERK1/2-mediated pathway. Furthermore, thymocytes from LEC rats also showed reduced ERK1/2 phosphorylation, which also suggests the regulation of ERK1/2 phosphorylation by PTPRK. Although the substrate for PTPRK resulting in altered ERK1/2 phosphorylation is not known, there are several reports showing that PTPRK directly dephosphorylates β-catenin [13] and epidermal growth factor (EGF) receptor [14]. Although EGF receptor-mediated signaling controls ERK1/2, there is no evidence showing that EGF receptor controls $CD4^+$ T cell development. Furthermore, the β-catenin pathway is involved in T cell development but does not selectively contribute to $CD4^+$ T cell development [15]. Therefore, PTPRK might dephosphorylate other substrates that are associated with $CD4^+$ T cell development through MAPK activation. The identification of such substrates would help us to better understand $CD4^+$ T cell development. In addition, our present data indicate that PTPRK positively regulates the ERK1/2 pathway. Although ERK1/2 is involved in $CD4^+$ T cell development, it is possible that PTPRK controls other signaling pathways independent of ERK1/2, which also contributes to $CD4^+$ T cell development.

$CD4^+$ T cell development is tightly regulated by many steps, including lineage commitment, progression and cell survival. $CD8^+$ T cell development in LEC rats is unaffected, which suggests that PTPRK is involved in the lineage progression of $CD4^+$ T cells rather than lineage choice between CD4/CD8 T cells. ERK1/2 is also involved in the lineage progression of $CD4^+$ T cells but not the CD4/CD8 lineage choice. Therefore, PTPRK/ERK might control the lineage progression of $CD4^+$ T cells, although ERK1/2 also has some role in $CD8^+$ T cell development.

The present studies demonstrate that PTPRK controls ERK1/2-mediated signaling. This novel interaction of PTPRK and ERK1/2 might provide new insight into not only $CD4^+$ T cell development but also other ERK1/2-mediated cellular responses.

Acknowledgments

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Notch signaling drives IL-22 secretion in CD4$^+$ T cells by stimulating the aryl hydrocarbon receptor

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CD4$^+$ helper T (Th) cells differentiate toward distinct effector cell lineages characterized by their distinct cytokine expression patterns and functions. Multiple Th cell populations secrete IL-22 that contributes to both protective and pathological inflammatory responses. Although the differentiation of IL-22-producing Th cells is controlled by the aryl hydrocarbon receptor (AhR), little is known about the regulatory mechanisms inducing physiological stimulators for AhR. Here, we show that Notch signaling enhances IL-22 production by CD4$^+$ T cells by a mechanism involving AhR stimulation. Notch-mediated stimulation of CD4$^+$ T cells increased the production of IL-22 even in the absence of STAT3. CD4$^+$ T cells from RBP-J-deficient mice had little ability to produce IL-22 through T cell receptor-mediated stimulation. RBP-J-deficient mice were highly susceptible to the detrimental immunopathology associated with ConA-induced hepatitis with little IL-22 production by CD4$^+$ T cells. Exogenous IL-22 protected RBP-J-deficient mice from ConA-induced hepatitis. Notch signaling promoted production of endogenous stimulators for AhR, which further augmented IL-22 secretion. Our studies identify a Notch–AhR axis that regulates IL-22 expression and fine-tunes immune system control of inflammatory responses.

inflammation | cytokine

Interleukin (IL)-22 belongs to the IL-10 superfamily of cytokines and exhibits potent proinflammatory and anti-inflammatory properties (1–4). IL-22 is highly expressed in IFN-γ (Th1) and IL-17-producing CD4$^+$ T helper cells (Th17), and recent studies have demonstrated that dendritic cells, NK cells, and lymphoid tissue-inducer cells also produce IL-22 (4). Although IL-6 and TGF-β are required for Th17 development, IL-6 induces IL-22 production, whereas TGF-β has a suppressive effect (5). Recent studies have demonstrated that ligation of the aryl hydrocarbon receptor (AhR) drives Th17 differentiation and IL-22 expression (6–8), although exogenous or endogenous ligands for AhR and inducers for endogenous ligands remain to be clarified. Furthermore, it also remains to be clarified if the same regulatory mechanism controls IL-22 expression in distinct Th subsets, including Th1 and Th17, and other cells.

Notch is an evolutionally conserved molecule and controls cell fate decision in a variety of cells (9, 10). Notch molecules are cleaved in the transmembrane region by γ-secretase after interaction with their ligands, followed by intracellular domain translocation into the nucleus (9, 10). We and other groups have demonstrated that Notch signaling controls the effector functions of both CD4$^+$ and CD8$^+$ T cells (11–15).

In this report we investigated the possibility that Notch controls IL-22 expression in CD4$^+$ T cells and found that deletion of RBP-J impaired IL-22 production in CD4$^+$ T cells. Notch signaling was able to up-regulate IL-22 even in STAT3-deficient T cells. This up-regulation of IL-22 was due to Notch-mediated production of AhR stimulators. These data indicate a regulatory mechanism of the immune system through IL-22 production by the Notch-AhR axis.

Results

Overexpression of Intracellular Domain of Notch in CD4$^+$ T Cells Upregulates IL-22 Independent of Th17 Differentiaiton. We searched by DNA microarray for genes up-regulated by the transduction of the intracellular domain of Notch2 (N2ICD), the active form of Notch2, in DO11.10 T cell hybridomas. This analysis identified a strong induction of IL-22 in N2ICD-transduced cells. To confirm IL-22 induction by Notch signaling, we introduced the intracellular domain of Notch1 (N1ICD), Notch2 (N2ICD), and Notch3 (N3ICD) into primary splenic T cells stimulated with anti-CD3 mAb and examined the expression of IL-22 in CD4$^+$ T cells by real-time PCR after 48 h. CD4$^+$ T cells transduced with each Notch intracellular domain had significantly increased expression of IL-22 compared with control mock-transduced cells (Fig. 1*A*). To confirm the contribution of CD4$^+$ T cells in our system, we checked the purity of CD4$^+$ T cells after MACS purification. Approximately 97% cells were positive for CD4 after MACS purification (Fig. S1*A*). RBP-J-deficient T cells did not show any increase in IL-22 production when transduced with N2ICD (Fig. S1*B*), indicating that Notch-mediated IL-22 up-regulation depends on RBP-J. We also tested the expression of other cytokines like IL-17A, IFN-γ, IL-4, TNF-α, and IL-10, along with other IL-10 family members, after transducing N2ICD into splenic T cells. Notch signaling only up-regulated the expression of IL-4 and IFN-γ (Fig. S1*C*), suggesting a specific effect of Notch on particular cytokines' expression rather than a nonspecific regulatory effect of Notch on T cell effector functions. We also checked the expression of transcription factors associated with different helper T cell differentiation and found that both T-bet and Gata-3 expression was up-regulated after N2ICD transduction, whereas RORγt expression that is required for Th17 differentiation (16) was unchanged (Fig. S1*D*). These data indicate that Notch functions to specifically up-regulate IL-22 expression without affecting Th17 differentiation and other IL-10 family cytokines, excluding a possibility that Notch controls IL-22 expression by nonspecific regulatory effect on T cell effector functions.

Stimulation of CD4$^+$ T Cells by Notch Ligand Upregulates IL-22 in CD4$^+$ T Cells. To clarify the direct contribution of Notch signaling in CD4$^+$ T cells in terms of IL-22 secretion, we purified CD4$^+$ T cells 48 h after transduction of total spleen cells or purified CD4$^+$

Author contributions: K.Y. designed research; M.S.A., Y.M., A.K., and K.K. performed research; K.T. and T.Y. contributed new reagents/analytic tools; M.S.A. and Y.M. analyzed data; and M.S.A. and K.Y. wrote the paper.

The authors declare no conflict of interest.

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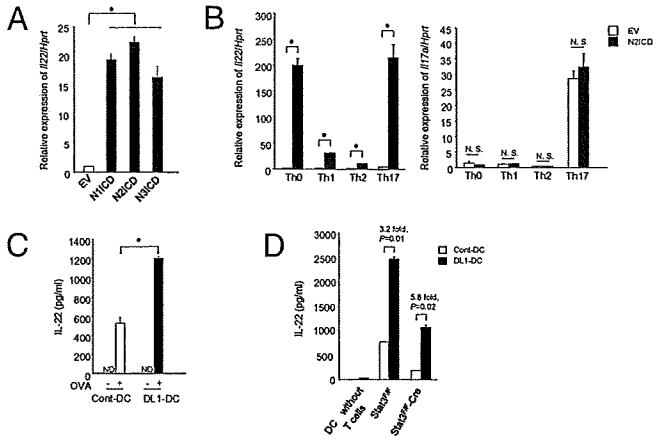

Fig. 1. Enforced expression of Notch in T cells induces IL-22. (*A*) Total spleen cells from C57BL/6 mice were stimulated with soluble anti-CD3 mAb (1 μg/mL) for 24 h and transduced with a retrovirus carrying N1ICD, N2ICD, N3ICD, or a control virus. Cells were further stimulated with anti-CD3 mAb (1 μg/mL) for 48 h. Then, the expression of *Il22* in MACS-enriched CD4$^+$ T cells was analyzed by real-time PCR. (*B*) Total spleen cells were stimulated with anti-CD3 mAb in Th0, Th1, Th2, and Th17 conditions for 24 h and then transduced with N2ICD or control virus. After 48 h of further culture under the same conditions, *Il22* and *Il17a* expression in MACS-enriched CD4$^+$ T cells was measured by real-time PCR. (*C*) ELISA for the detection of IL-22 secretion from naive CD4$^+$ T cells (CD4$^+$CD62L$^+$) isolated from OT-II TCR transgenic mice stimulated with OVA peptide-pulsed Cont-DC (open) or DL1-DC (filled) for 3 days. (*D*) ELISA for the detection of IL-22 secretion from naive CD4$^+$ T cells isolated from STAT3$^{F/F}$ or STAT3$^{F/F}$-Cre transgenic mice stimulated with allogenic Cont-DC (open) or DL1-DC (filled) prepared from BALB/c mice. *, $P < 0.05$, indicates a statistically significant difference. Data are representative of at least four independent experiments. N.D., not detected; N.S., not significant.

T cells with N2ICD or EV and measured the expression of IL-22 by real-time PCR. Similar to experiments with total splenocytes, we found that N2ICD transduction caused increased expression of IL-22 in purified naive CD4$^+$ T cells, although IL-22 expression by CD4$^+$ T cells was higher in the presence of APC compared with purified naive CD4$^+$ T cells (Fig. S2*A*). To identify the effector T cell type responding to Notch, we introduced N2ICD into primary spleen cells under Th0, Th1, Th2, and Th17-promoting conditions. We found that expression of IL-22 in CD4$^+$ T cells was increased by Notch signaling in all culture conditions (Fig. 1*B Left*) but not IL-17A (Fig. 1*B Right*). In particular, the effect of Notch signaling was great under Th0 or Th17 condition. The expression of IL-17 was comparable among Th0, Th1, and Th2 conditions. Those data again suggest that Notch-mediated IL-22 production is not necessarily dependant on Th17 differentiation. We also checked the expression of IL-4 and IFN-γ in all culture conditions and found induced expressions of both cytokines after N2ICD transduction (Fig. S2*B*). The expression of Notch1 and Notch2 was comparable for each Th condition (Fig. S2*C*).

We further examined the effect of Notch signaling on IL-22 expression by directly stimulating T cells with Notch ligand. To induce Notch signaling in CD4$^+$ T cells, we stimulated naïve OT-II TCR transgenic T cells with bone marrow-derived, OVA peptide-pulsed dendritic cells (BMDCs) transduced with Delta-like 1 (DL1-DC) for 3 days. DL1-DC-stimulated OT-II T cells secreted more IL-22 in the culture supernatant than when mock-transduced DCs (Cont-DC) were used (Fig. 1*C*). The secretion of IFN-γ was also induced with DL1-DC-stimulated OT-II T cells (Fig. S3*A*). We also stimulated naive OT-II CD4$^+$ T cells with Cont-DC or DL1-DC under neutral, Th1, Th2, or Th17 conditions for 3 days and found increased expression of IL-22 by DL1-DC in all culture conditions (Fig. S3*B*). These data indicate that Notch-mediated stimulation of CD4$^+$ T cells up-regulates IL-22 expression under any helper T cell culture condition.

Notch Signaling Controls IL-22 Expression in CD4$^+$ T Cells in the Absence of STAT3. Th17 is one T cell lineage that expresses IL-22, although Th1, γδ T cells, NK cells, NKT cells, and DC can also secrete IL-22 (17). IL-6 and TGF-β are essential cytokines for Th17 differentiation, and IL-23 is responsible for the survival and expansion of Th17 cells (17). Therefore, we examined whether these cytokines took part in Notch signaling-induced IL-22 production. For this purpose, we measured IL-22 secretion during an allo-response, coculturing DL1-DC or Cont-DC prepared from BALB/c mice with naive CD4$^+$ T cells from STAT3$^{flox/flox}$ (STAT3$^{F/F}$) or STAT3$^{flox/flox}$ crossed with lck-Cre transgenic (STAT3$^{F/F}$-Cre) mice (18). IL-22 production was reduced in STAT3$^{F/F}$-Cre CD4$^+$ T cells when either Cont-DC or DL1-DC was used as stimulators, indicating that STAT3 is important for IL-22 production (Fig. 1*D*). We further found that DL1-DC could up-regulate IL-22 secretion even from STAT3$^{F/F}$-Cre CD4$^+$ T cells (Fig. 1*D*). This value is significantly higher than that of the Cont-DC-stimulated STAT3$^{F/F}$-CD4$^+$ T cells. Hence, these data indicate that forced Notch signaling can induce IL-22 production from CD4$^+$ T cells with mechanisms distinct from STAT3 signaling pathway.

RBP-J Deficiency in CD4$^+$ T Cells Impairs IL-22 Production. RBP-J is a transcription factor essential for Notch signaling (9). Next, we investigated IL-22 production in CD4$^+$ T cells in the absence of RBP-J. When CD4$^+$ T cells from RBP-J$^{flox/flox}$ mice crossed with both CD4-Cre and OT-II TCR transgenic (RBP-J$^{F/F}$-Cre OT-II) mice were stimulated with OVA peptide presented by BMDCs, their IL-22 secretion was impaired in contrast to RBP-J$^{+/+}$ mice crossed with CD4-Cre and OT-II TCR transgenic (RBP-J$^{+/+}$-Cre OT-II) mice (Fig. 2*A*). RBP-J deficiency also caused decreased secretion of IFN-γ in this system (Fig. S3*C*). We did not see any difference between T cells from RBP-J$^{+/+}$-Cre OT-II and RBP-J$^{F/F}$-Cre OT-II mice in their proliferative responses to peptide-pulsed DCs (Fig. S3*D*).

To further confirm the contribution of Notch signaling in BMDC coculture system, we stimulated naive CD4$^+$ T cells isolated from OT-II TCR transgenic mice with OVA-pulsed BMDCs in the presence of γ-secretase inhibitor. The γ-secretase inhibitor decreased IL-22 production from CD4$^+$ T cells (Fig. S3*E*). These data indicate that Notch signaling controls IL-22 secretion from CD4$^+$ T cells through γ-secretase-mediated cleavage of Notch and RBP-J.

To further examine the involvement of RBP-J in IL-22 production in vivo, we immunized RBP-J$^{flox/flox}$ mice crossed with CD4-Cre transgenic (RBP-J$^{F/F}$-Cre) mice with OVA emulsified in complete Freund's adjuvant (CFA) and examined IL-22 expression in CD4$^+$ T cells 7 days after immunization. IL-22 expression was highly impaired in RBP-J$^{F/F}$-Cre mice compared with control RBP-J$^{+/+}$-Cre mice (Fig. 2*B*), whereas IL-17A expression was intact (Fig. S4*A*). These data also reveal that Notch signaling controls IL-22 expression independent of Th17 differentiation. We also investigated the proliferation of CD4$^+$ T cells in OVA-immunized mice, and there was no difference between RBP-J$^{F/F}$-Cre and RBP-J$^{+/+}$-Cre mice (Fig. 2*C*). To test which Notch receptor contributes to IL-22 production in vivo, we immunized Notch1$^{F/F}$-Cre (Notch1$^{flox/flox}$ mice crossed with CD4-Cre transgenic mice), Notch2$^{F/F}$-Cre (Notch2$^{flox/flox}$ mice crossed with CD4-Cre transgenic mice), and RBP-J$^{F/F}$-Cre mice with OVA emulsified in CFA and examined IL-22 expression in CD4$^+$ T cells 7 days after immunization. Although IL-22 expression was highly impaired in RBP-J$^{F/F}$-Cre mice compared with control RBP-J$^{+/+}$-Cre mice, IL-22 expression was not impaired under each Notch receptor deficiency (Fig. S4*B*). Taken together, including both in vitro and in vivo data, RBP-J is important for IL-22 production but not for IL-17A, indicating that Notch signaling controls IL-22 expression, although it remains unclear which Notch receptor is

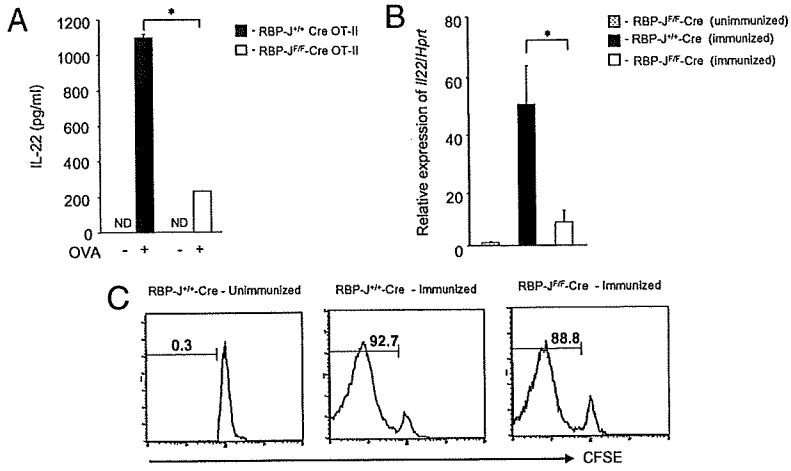

Fig. 2. Deficiency of Notch signaling in CD4$^+$ T cell impairs IL-22 expression. (*A*) Naive CD4$^+$ T cells isolated from RBP-J$^{+/+}$-Cre OT-II (filled) or RBP-J$^{F/F}$-Cre OT-II (open) mice were stimulated with OVA peptide-pulsed BMDCs for 3 days. IL-22 concentrations in the supernatants were evaluated by ELISA. (*B*) CD4$^+$ T cells were isolated from RBP-J$^{+/+}$-Cre (filled) and RBP-J$^{F/F}$-Cre (open) mice 7 days after immunization with OVA emulsified in CFA. As a control, CD4$^+$ T cells from unimmunized RBP-J$^{F/F}$-Cre mice (dot) were isolated. *Il22* expression in CD4$^+$ T cells was evaluated by real-time PCR. (*C*) CFSE-labeled splenic T cells from RBP-J$^{+/+}$-Cre OT-II or RBP-J$^{F/F}$-Cre OT-II mice were transferred into C57BL/6 Thy1.1 mice that were immunized with OVA protein emulsified in CFA soon after T cell transfer. CFSE dilution was evaluated after gating on CD4$^+$ Thy1.2$^+$ cells 7 days after OVA immunization. *, $P < 0.05$, indicates a statistically significant difference. Data in Fig. 2 are representative of at least four independent experiments.

involved. These data also prove that the expression of IL-17 and IL-22 are differentially regulated.

RBP-J-Deficient Mice Have High Susceptibility to ConA-Induced Hepatitis. IL-22 is a pivotal cytokine in several inflammatory diseases, although its role remains controversial (3). That is, it is proinflammatory in psoriasis (5) and anti-inflammatory in irritable bowel disease (19) and Con A (ConA)-induced hepatic injury (20). We employed the ConA-hepatitis model in RBP-J$^{F/F}$-Cre mice to further define the contribution of Notch in IL-22 secretion in the context of autoimmunity. We injected several doses of ConA into mice and quantified the severity of hepatitis 48 h after injection. Both RBP-J$^{+/+}$-Cre and RBP-J$^{F/F}$-Cre mice had similar hepatic damage in response to high-dose (30 μg/g) injection of ConA. However, only RBP-J$^{F/F}$-Cre mice exhibited hepatic injury, including severe inflammation and bleeding, after a relatively low-dose (10 μg/g) ConA injection that did not induce hepatitis in control RBP-J$^{+/+}$-Cre mice (Fig. 3*A*). This increased susceptibility to low-dose ConA-induced hepatitis in RBP-J$^{F/F}$-Cre mice was also confirmed by the elevated liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Fig. 3*B*). We examined CD4$^+$ T cell proliferation in vivo, and there was no dif-

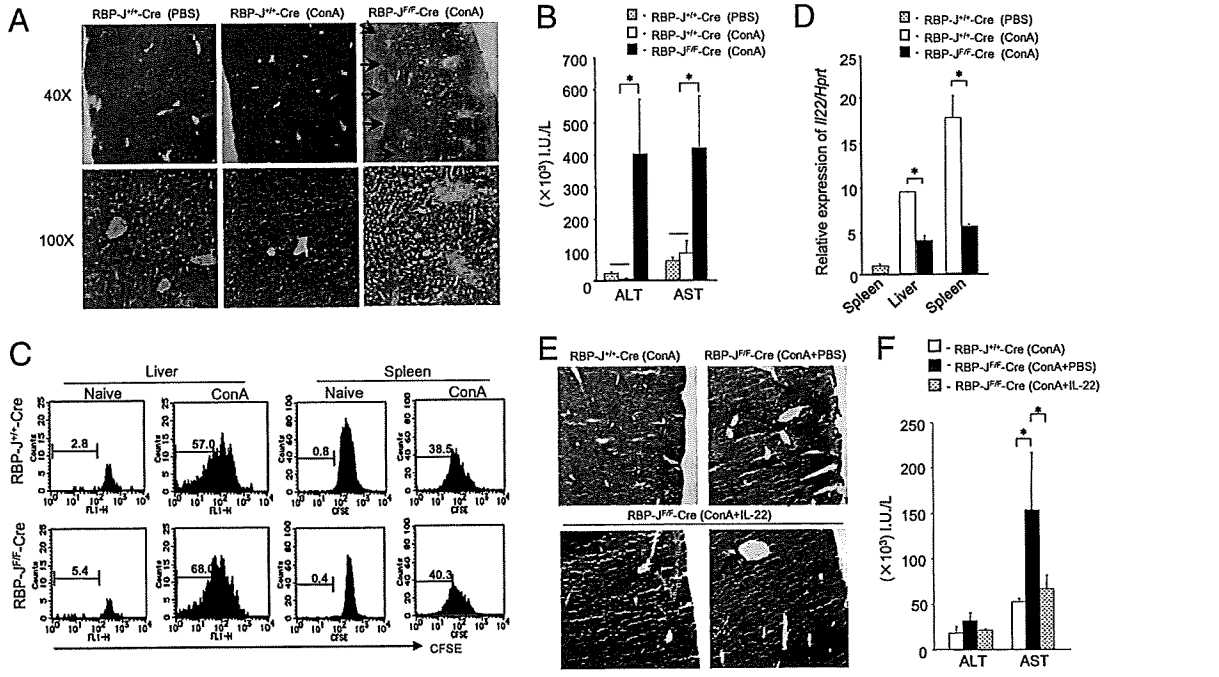

Fig. 3. Notch deficiency in T cells results in susceptibility to ConA-induced hepatitis. The RBP-J$^{+/+}$-Cre or RBP-J$^{F/F}$-Cre mice received ConA (10 μg/g) and/or IL-22 (2 μg per mouse). As a control, RBP-J$^{F/F}$-Cre mice received PBS. (*A*) Liver sections 48 h after ConA injection were stained with hematoxylin and eosin. Red arrows indicate bleeding. (*B*) The ALT and AST levels in the serum of each mouse were measured. (*C*) CFSE-labeled total spleen cells from RBP-J$^{+/+}$-Cre or RBP-J$^{F/F}$-Cre mice were transferred into C57BL/6 Thy 1.1 mice, and transferred mice received ConA. Lymphocytes were purified from liver and spleen by Lympholyte M 48 h after ConA injection, and CFSE dilution was plotted after gating on CD4$^+$Thy1.2$^+$ cells. (*D*) RBP-J$^{+/+}$-Cre or RBP-J$^{F/F}$-Cre mice received ConA (10 μg/g). CD4$^+$ T cells were purified from livers and spleens 48 h after ConA injection by Lympholyte M and MACS beads and *Il22* mRNA was measured by real-time PCR. (*E*) RBP-J$^{+/+}$-Cre and RBP-J$^{F/F}$-Cre mice received ConA (10 μg/g) with IL-22 (2 μg) or PBS. Liver sections 48 h after ConA and IL-22 injection were stained with hematoxylin and eosin. Red arrows indicate bleeding, and black arrow indicates cell infiltration. (*F*) The ALT and AST levels in the serum of each mouse were measured. *, $P < 0.05$, indicates a statistically significant difference. Data in Fig. 3 are representative of at least four independent experiments.

ference in ConA-induced T cell proliferation between the two groups (Fig. 3*C*). The IL-22 transcription was highly impaired in $CD4^+$ T cells from RBP-$J^{F/F}$-Cre mice in both liver and spleen (Fig. 3*D*). However, we found that the expression of other inflammatory cytokines in liver and splenic $CD4^+$ T cells of ConA-injected mice, such as IFN-γ, IL-4, TNF-α, IL-17A, and the anti-inflammatory cytokine IL-10, was comparable between the two groups (Fig. S5*A*). Furthermore, expression of FasL was also comparable between two groups (Fig. S5*B*). To know if non-$CD4^+$ T cells also express IL-22, we checked the expression of IL-22 in NK, NKT, or $CD8^+$ T cells. We injected ConA (10 μg/g) to wild-type C57BL/6 mice and after 48 h of injection, we sorted CD4, CD8, NKT (NK1.1 and CD3 double positive), and NK (CD3 negative and NK1.1 positive) cells from liver and checked IL-22 expression by real-time PCR. As expected, IL-22 expression was only up-regulated in $CD4^+$ T cells (Fig. S5*C*), suggesting that $CD4^+$ T cells provide protection in ConA-induced acute hepatitis by secreting IL-22. These results suggest that the increased susceptibility to ConA-induced hepatic injury in RBP-$J^{F/F}$-Cre mice might be due to decreased production of protective IL-22 by $CD4^+$ T cells.

We next tested whether exogenous IL-22 administration is able to cure ConA-hepatitis in RBP-$J^{F/F}$-Cre mice by compensating for low or absent endogenous IL-22. The low-dose ConA injection did not induce any pathological change in RBP-$J^{+/+}$-Cre mice, whereas the injection of ConA induced severe bleeding in the liver in RBP-$J^{F/F}$-Cre mice (Fig. 3*E Upper*). When we administered recombinant IL-22 along with ConA in RBP-$J^{F/F}$-Cre mice, 25% of the mice showed mild bleeding (Fig. 3*E Lower Left*) and 75% had no bleeding at all in the liver (Fig. 3*E Lower Right*). Furthermore, serum AST levels were also significantly decreased by IL-22 injection in RBP-$J^{F/F}$-Cre mice (Fig. 3*F*). Hence, the ConA-induced IL-22 production depends on Notch signaling, as evidenced by the ability of IL-22 to protect RBP-J-deficient mice from liver injury.

Notch Controls IL-22 by Affecting a Signaling Pathway Through AhR. There is no consensus RBP-J binding element in the 800 bp proximal promoter of IL-22 conserved between human and mouse, suggesting that Notch indirectly regulates the induction of IL-22. Recent reports describe the essential role of the AhR in IL-22 production (6–8). Therefore, we checked whether IL-22 production due to Notch signaling depended on AhR signaling. Blocking AhR signaling by the AhR antagonist CH-223191 (2-Methyl-2H-pyrazole-3-carboxilic acid-(2-methyl-4-*o*-tolyl-azophenyl)-amide) (20 μM) in N2ICD-transduced T cells preferentially decreased IL-22 expression (Fig. 4*A Left*) and was concentration dependent (Fig. S6*A*), whereas IL-17A expression was unaffected (Fig. 4*A Right*). Next, we tested whether the expression of AhR was influenced by N2ICD transduction and found that AhR expression was comparable with mock transduction (Fig. 4*B*). We also examined AhR expression under different effector T cell culture conditions and found that although AhR expression was highest under Th17 conditions, N2ICD did not further up-regulate AhR expression under any of the culture conditions (Fig. S6*B*). These data do not suggest that Notch-mediated IL-22 production depends on AhR expression itself. Similarly, AhR antagonism significantly decreased the expression of IL-22 in N2ICD-transduced DO11.10 T cell hybridoma cells without T cell receptor-mediated signaling (Fig. 4*C*).

Notch Signaling Allows $CD4^+$ T Cells To Produce AhR Stimulators. According to the results that IL-22 induction by Notch signaling was inhibited by AhR antagonism, and that Notch signaling does not up-regulate AhR, we hypothesized that Notch signaling might increase an endogenous AhR agonist. To test this hypothesis, we harvested the culture supernatant from total T cells transduced with N2ICD (N2ICD-sup) and added dilutions of this supernatant during the stimulation of $CD4^+$ T cells. The $CD4^+$ T cells with N2ICD-sup significantly increased IL-22 expression compared with that of control supernatant (EV-sup), and in a dose-dependent manner (Fig. 4*D Left*). The up-regulation of IL-22 by adding N2ICD-sup was suppressed by an AhR antagonist. Indeed, N2ICD-sup also increased expression of Cyp1a1, the typical downstream target gene of AhR signaling (Fig. 4*D Right*), indicating that N2ICD-sup contained a factor(s) competent to induce AhR signaling. Because overexpression of N2ICD in $CD4^+$ T cells induced both IL-4 and IFN-γ expression (Figs. S1*C* and 2*B*), we checked whether N2ICD-sup affects IL-4 and IFN-γ expression in $CD4^+$ T cells. We found that N2ICD-sup did not affect the

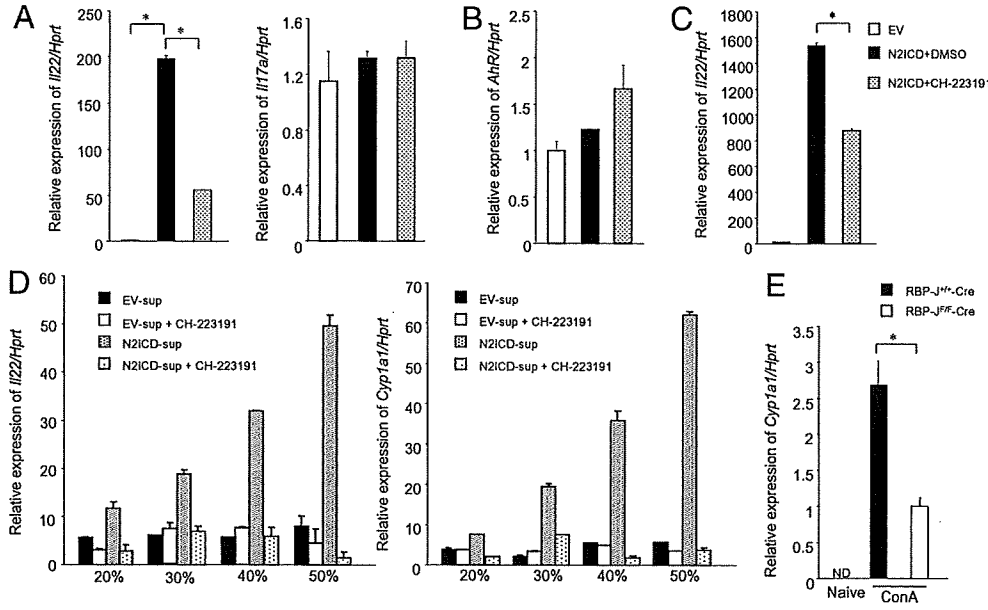

Fig. 4. Notch signaling induces AhR stimulators. Total spleen cells were stimulated with anti-CD3 mAb for 24 h and transduced with a retrovirus carrying N2ICD or a control vector. After infection, cells were restimulated with anti-CD3 mAb for 48 h in the presence (filled) or absence (open) of an AhR antagoinst. Then *Il22* and *Il17a* (*A*) or *AhR* expression in $CD4^+$ T cells (*B*) was measured by real-time PCR. (*C*) The expression of *Il22* after retroviral transduction of N2ICD (filled or dot) or EV (open) in DO11.10 T cells hybridoma in the presence (filled) or absence (dot) of the AhR antagonist was examined by real-time PCR. (*D*) The supernatant from total spleen cells transduced with N2ICD (dot or shaded) or EV (open or filled) was collected 72 h after initial stimulation. $CD4^+$ T cells from C57BL/6 mice were stimulated with anti-CD3 mAb in the presence of different concentrations of supernatant and in the presence (open, shaded) or absence (filled, dot) of an AhR antagonist. The expression of *Il22* (*Left*) and *Cyp1a1* (*Right*) in mature $CD4^+$ T cells was tested 48 h after initial stimulation. (*E*) The RBP-$J^{+/+}$-Cre or RBP-$J^{F/F}$-Cre mice received ConA (10 μg/g) and the expression of *Cyp1a1* in liver $CD4^+$ T cells purified by MACS beads 48 h after ConA injection was measured by real-time PCR. *, $P < 0.05$, indicates a statistically significant difference. Data in Fig. 4 are representative of at least three independent experiments.

expression of both cytokines (Fig. S6*C*), suggesting that Notch-AhR axis is not involved in Th1 or Th2 differentiation and specifically contributes to IL-22 secretion.

To identify the effector T cell culture conditions that best promote the production of AhR stimulators, we used N2ICD-transduced total splenocytes supernatants from different effector T cell culture conditions to stimulate spleen cells. We found that using the supernatants collected from N2ICD-transduced Th0 and Th17 conditions resulted in the highest IL-22 expression (Fig. S6*D*). We also considered the issue of whether DC present in the culture could induce AhR stimulators. We introduced N2ICD in total splenocytes and purified $CD4^+$ T cells and cultured for 48 h. The collected supernatants were used to stimulate purified $CD4^+$ T cells in the presence of anti-CD3 mAb. We observed increased expressions of both IL-22 and Cyp1a1 when N2ICD supernatant was used (Fig. S6*E*). This effect was stronger in the presence of APC than in the absence of APC (Fig. S6*E*), probably because of efficient T cell stimulation in the presence of APC.

To identify AhR stimulator production in vivo, we purified $CD4^+$ T cells from livers 48 h after ConA injection of RBP-$J^{+/+}$-Cre and RBP-$J^{F/F}$-Cre mice and restimulated such cells with anti-CD3 mAb or left unstimulated for 24 h. Finally, we used such supernatant to restimulate total splenic $CD4^+$ T cells for 48 h in the presence of anti-CD3 mAb (Fig. S6*F*). The supernatant of purified $CD4^+$ T cells of RBP-$J^{+/+}$-Cre mice had induced Cyp1a1 expression and IL-22 (Fig. S6*F*). The supernatant collected from restimulated cells of RBP-$J^{F/F}$-Cre mice had no effect on either Cyp1a1 or IL-22 (Fig. S6*F*). To confirm this finding in vivo, we measured Cyp1a1 expression in $CD4^+$ T cells from livers 48 h after ConA injection in RBP-$J^{+/+}$-Cre and RBP-$J^{F/F}$-Cre mice. The expression of Cyp1a1 was lower in T cells from RBP-$J^{F/F}$-Cre mice than in those from RBP-$J^{+/+}$-Cre mice (Fig. 4*E*). These results indicate that Notch signaling up-regulated IL-22 expression by inducing a natural ligand that resulted in AhR signaling.

We tested up-regulated genes in T cell hybridoma transduced with N2ICD by DNA microarray and evaluated genes involved in cell development, signal transduction, and metabolism (Table S1). However, we could not find any molecules related to tryptophan metabolism. Therefore, we evaluated whether the 6-formylindolo [3,2-b] carbazole (FICZ), a tryptophan photoproduct and an only known endogenous ligand for AhR signaling, is involved in Notch-mediated IL-22 production (21). Hence, we used Sep-Pak Plus C_{18} cartridges to track FICZ activity in our system because this cartridge can trap FICZ very efficiently (21). We stimulated total spleen cells with N2ICD supernatant, flow-through, and eluate of a Sep-Pak Plus C_{18} cartridge in conjunction with anti-CD3 mAb. The expression of both IL-22 and Cyp1a1 was increased by N2ICD-transduced supernatant and flow-through compared with that of EV (Fig. S7*A*). In contrast, we could not detect any such activity in any elute fraction, suggesting that FICZ is not involved in our system. To examine whether the AhR stimulator is a heat labile protein, we heated the flow-through to 95 °C for 10 min and used this flow-through to restimulate total spleen cells. We found that heating the flow-through collected from N2ICD-transduced supernatant abrogated the up-regulation of IL-22 and Cyp1a1 (Fig. S7*B*), suggesting that Notch signaling induces the production of heat labile molecules as AhR stimulators.

Discussion

Several studies have highlighted the importance of IL-22 in inflammatory responses (20, 22, 23). Likewise, the role of AhR in Th17 cells producing IL-22 has recently received much attention (6, 7), although the environmental and endogenous agents that control AhR-mediated IL-22 expression remain unclear. The present data demonstrate that Notch signaling is crucial for IL-22 production from $CD4^+$ T cells, which depends on the production of endogenous stimulators of AhR signaling, but not STAT3. Blockade of Notch signaling in T cells increased susceptibility for ConA-induced inflammatory hepatic injury due to dramatically reduced IL-22 production, stressing the crucial role of Notch signaling in IL-22 production. Because there are many endogenous molecules able to activate AhR (24), the physiological ligands for AhR in terms of Notch-mediated IL-22 production will be an interesting issue to be addressed in the context of regulating inflammatory responses.

Mounting evidence supports that IL-22 production depends on IL-23 or IL-6 (17). The present study revealed that Notch signaling is able to up-regulate IL-22 production from $CD4^+$ T cells even in the absence of STAT3 or up-regulation of RORγt, which are crucial for IL-6 signaling or Th17 differentiation, respectively (16). These data indicate that Notch-mediated IL-22 production is independent of Th17 differentiation, which also excludes the possibility that Notch-mediated IL-22 production results from nonspecific regulatory roles of Notch on T cell effector functions. As for the relationship between Notch and STAT3 signaling, our data suggest that IL-22 production is controlled by several signaling pathways, although each pathway might converge. It would be interesting to test whether STAT3 or Notch-AhR axis-mediated IL-22 production has distinct roles in immune responses, or whether distinct cell types use a particular signaling pathway to induce IL-22. In addition, we found in this study that overexpression among active Notch1, Notch2, or Notch3 in $CD4^+$ T cells is able to up-regulate IL-22, indicating that any Notch signaling is able to induce IL-22. We did not observe decreased IL-22 production in Notch1- or Notch2-deficient mice, although it is possible that Notch1 and Notch2 compensate each other.

Previous studies have demonstrated that Notch signaling controls many aspects of effector function of not only $CD4^+$ but also $CD8^+$ T cells (10–15). We and other groups have demonstrated that Notch signaling controls cytolytic effector functions in $CD8^+$ T cells (13, 25), and recent studies have revealed that IL-22 is also produced by $CD8^+$ T cells (17). Because the studies described in this report used CD4-Cre transgenic mice to delete the RBP-J gene, both $CD4^+$ and $CD8^+$ T cells lack the RBP-J gene in our system because of thymic expression of Cre in $CD4^+CD8^+$ T cells. Therefore, we cannot completely deny the possibility that Notch also controls IL-22 production from $CD8^+$ T cells, which may contribute to the susceptibility of ConA-induced hepatitis. However, we think such a possibility is unlikely because we showed that $CD8^+$ T cells did not up-regulate IL-22 after ConA injection. In addition, previous papers reported the contribution of NKT cells in the pathogenesis of ConA-induced hepatitis (26, 27). Although those studies revealed that the possible contribution of NKT cells is more likely inflammatory rather than anti-inflammatory, it would be important to determine whether Notch controls IL-22 production by NKT cells.

Present studies revealed that overstimulation of Notch in $CD4^+$ T cells is able to up-regulate IL-22 under neutral, Th1, Th2, and Th17 promoting culture conditions. Those data suggest at least three possibilities. The first possibility is that Notch signaling is able to help express IL-22 even in fixed helper T cells. The second one is that Notch signaling up-regulates IL-22 in a small fraction of naïve $CD4^+$ T cells that did not receive enough cytokine signaling to skew toward each helper cell lineage. The third one is that Notch signaling helps differentiation of new type of helper cells such as IL-22 and IFN-γ or IL-22 and IL-4 double producer. To analyze these possibilities in the future, it is necessary to examine cytokines and transcription factors in a single cell, by establishing specific antibodies useful for flow cytometoric analysis.

Our present studies have demonstrated that Notch-mediated stimulation of $CD4^+$ T cells helps produce AhR stimulators. The AhR stimulators interact with $CD4^+$ T cells, which up-regulates IL-22 in $CD4^+$ T cells as we showed in ConA-induced hepatitis model, although AhR stimulators might also interact with other cells. We found that a known endogenous AhR ligand, FICZ, is not involved in Notch-induced AhR stimulation in our model system, although heat labile proteins do appear to be candidates for AhR stimulators. The identification of such endogenous AhR stimulators would contribute not only to our understanding of the mechanism how Notch controls

IL-22 production in T cells, but also to our understanding of the other physiological roles of AhR in cellular responses.

Notch is crucial for a variety of behaviors in cells, including cell proliferation, tumorigenesis, cell fate decisions, and embryogenesis. It is thought that AhR-mediated environmental signals affect tumorigenesis and cell differentiation (28). For instance, overstimulation of AhR by environmental signals might enhance physiological Notch-mediated AhR stimulation, which would contribute to Notch over-activating phenotypes such as tumor cells or aberrant cell fate decisions. Therefore, the newly identified Notch-AhR axis would suggest not only a unique regulatory mechanism for inflammatory responses, but also a close link between the broad regulation of Notch-mediated cell differentiation and environmental signals. Our findings may also advocate for the manipulation of AhR pathway components as a means to modulate cell activation or as a therapy for Notch-mediated tumorigenesis.

Methods

Mice. Female C57BL/6 mice (6–8 weeks old) and Thy1.1 C57BL/6 mice were obtained from Japan SLC or The Jackson Laboratory, respectively). RBP-J$^{flox/flox}$ mice crossed with CD4-Cre mice (15, 29) were further crossed with OT-II TCR transgenic (Taconic) mice. STAT3$^{flox/flox}$ mice and STAT3$^{flox/flox}$ crossed with Ick-Cre transgenic mice were reported (18). Mice were housed in the Animal Research Center of the University of Tokushima under specific pathogen-free conditions, and all animal work was approved by the Animal Research Committee of the University of Tokushima.

Cell Culture. Naive CD4$^+$ T cells (CD4$^+$CD62L$^+$) were isolated from spleens by paramagnetic bead enrichment according to the manufacturer's protocol (Miltenyi Biotech). For purification of total CD4$^+$ cells, lymph node cells were incubated with anti-B220, anti-CD32/16, anti-CD11b, and anti-CD8 mAbs followed by incubation with anti-rat IgG-coated Dynabeads (Dynal). CD4$^+$ T cells were further purified by magnetic separation using biotin-conjugated anti-CD4 mAb and streptavidin microbeads (Miltenyi Biotech). DCs were generated from mouse bone marrow cells with GM-CSF (R&D Systems). Three days after the final retroviral infection, DCs were stimulated with LPS (1 μg/mL; Sigma). After overnight stimulation with LPS, CD11c$^+$ cells were isolated by magnetic separation with CD11c microbeads (Miltenyi Biotech). In some experiments, total spleen cells were labeled by 5-(and 6-) carboxyfluorescein diacetate succinamidyl ester (CFSE; Invitrogen) as described (13). For T cell stimulation, purified naive CD4$^+$ OT-II TCR transgenic T cells were stimulated with BMDCs pulsed with $OVA_{323-339}$ peptide (Abgent). In some BMDC and CD4$^+$ T cell coculture experiments, the 20 μM concentration of γ-secretase inhibitor (Calbiochem) was added. Retrovirus carrying N2ICD was used once to infect CD4$^+$ T cells 1 day after stimulation with anti-CD3 mAb. As for the different helper T cell differentiation conditions, we stimulated total spleen cells with anti-CD3 mAb with several combinations of cytokines and antibodies; Th1 [IL-12 (10 ng/mL) plus anti IL-4 mAb (10 μg/mL)], Th2 [IL-4 (30 ng/mL) plus anti-IL-12 mAb (10 μg/mL)] and Th17 [IL-6 (10 ng/mL) and TGF-β (2 ng/mL) plus anti IL-4 mAb (10 μg/mL) and anti IL-12 mAb (10 μg/mL)] throughout the culture.

Retroviral Infection. Total spleen cells were stimulated with anti-CD3 mAb (1 μg/mL) for 24 h and then retrovirus carrying N1ICD, N2ICD, or N3ICD were used once for infection. Finally, cytokine expression was analyzed 48 h after further stimulation with anti-CD3 mAb (1 μg/mL). The retroviral infection protocol under Th1, Th2, and Th17 culture conditions is the same as in neutral conditions. For naive CD4$^+$ T cells, infection procedures were the same as that of total splenocytes except that the cells were stimulated with plate-bound anti-CD3 mAb (1 μg/mL) instead of soluble anti-CD3 mAb. The DL1 gene was transduced into DCs via retroviral gene delivery repeated a total of three times (days 0, 1, and 2) as described (13).

ELISA. IL-22 in culture supernatants was measured by using an ELISA kit (R&D Systems). In the experiment shown in Fig. 1*D*, a FlowCytomix kit (Bender Medsystems) was used.

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Hematopoietic Stem Cell Transplantation for Familial Hemophagocytic Lymphohistiocytosis and Epstein–Barr Virus-Associated Hemophagocytic Lymphohistiocytosis in Japan

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Background. Post-transplant outcomes of hemophagocytic lymphohistiocytosis (HLH) patients were analyzed in Japan where Epstein–Barr virus (EBV)-associated severe forms are problematic. ***Methods.*** Fifty-seven patients (43 familial HLH [12 FHL2, 11 FHL3, 20 undefined], 14 EBV-HLH) who underwent stem cell transplantation (SCT) between 1995 and 2005 were enrolled based on the nationwide registration. ***Results.*** Fifty-seven patients underwent 61 SCTs, including 4 consecutive SCTs. SCTs were employed using allogeneic donors in 93% of cases (allo 53, twin 1, auto 3). Unrelated donor cord blood transplantation (UCBT) was employed in half of cases (21 FHL, 7 EBV-HLH). Reduced intensity conditioning was used in 26% of cases. The 10-year overall survival rates (median ± SE%) were 65.0 ± 7.9% in FHL and 85.7 ± 9.4% in EBV-HLH patients, respectively. The survival of UCBT recipients was >65% in both FHL and EBV-HLH patients. Three out of four patients were alive with successful engraftment after second UCBT. FHL patients showed a poorer outcome due to early treatment-related deaths (<100 days, seven patients) and a higher incidence of sequelae than EBV-HLH patients ($P = 0.02$). The risk of death for FHL patients having received an unrelated donor bone marrow transplant was marginally higher than that for a related donor SCT ($P = 0.05$) and that for UCBT ($P = 0.07$). ***Conclusions.*** EBV-HLH patients had a better prognosis after SCT than FHL patients. FHL patients showed either an equal or better outcome even after UCBT compared with the recent reports. UCB might therefore be acceptable as an alternate SCT source for HLH patients, although the optimal conditioning remains to be determined. Pediatr Blood Cancer 2010;54:299–306. © 2009 Wiley-Liss, Inc.

Key words: central nervous system disease; Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis; familial hemophagocytic lymphohistiocytosis; hematopoietic stem cell transplantation; reduced intensity conditioning; umbilical cord blood transplantation

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is an immunohematologic emergency, characterized by fever, cytopenias, hepatosplenomegaly, hyperferritinemia, and disseminated intravascular coagulopathy (DIC) [1,2]. HLH comprises primary form of familial hemophagocytic lymphohistiocytosis (FHL) and secondary form occurring in association with infections, malignancies, and rheumatic diseases. FHL has currently been classified into FHL1 linked to chromosome 9, FHL2 with *PRF1* mutation, FHL3 with *UNC13D* mutation, and FHL4 with *STX11* mutation, although more than half of patients have no mutations of these genes [1]. HLH could also be a presenting symptom in patients with the other inherited disorders including X-linked lymphoproliferative disease (XLP), Griscelli syndrome, Hermansky–Pudlak syndrome, Chediak–Higashi syndrome and primary immunodeficiency diseases. HLH accounts for the common basis of hypercytokinemia arising from excessive immune activation, in which activated lymphocytes and hemophagocytosing-macrophages without malignant morphology infiltrate into systemic organs, including the bone

Additional Supporting Information may be found in the online version of this article.

Abbreviations: BM, bone marrow; BMT, bone marrow transplantation; CB, cord blood; CBT, cord blood transplantation; CNS, central nervous system; CT, computed tomography; EBV-HLH, Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis; EEG, electroencephalography; FHL, familial hemophagocytic lymphohistiocytosis; HLH, hemophagocytic lymphohistiocytosis; PB, peripheral blood; SCT, hematopoietic stem cell transplantation; MRI, magnetic resonance imaging; OS, overall survival; SCT, hematopoietic stem cell transplantation; TRM, treatment-related mortality; RIC, reduced intensity conditioning; VOD, venoocclusive disease; XLP, X-linked lymphoproliferative disease/syndrome.

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