Notch signaling is an important regulator of type 2 immunity

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Notch ligands and receptors have been implicated in helper T cell (Th cell) differentiation. Whether Notch signals are involved in differentiation of T helper type 1 (Th1) cells, Th2 cells, or both, however, remains unresolved. To clarify the role of Notch in Th cell differentiation, we generated mice that conditionally inactivate Notch signaling in mature T cells. Mice that lack Notch signaling in CD4+ T cells fail to develop a protective Th2 cell response against the gastrointestinal helminth Trichuris muris. In contrast, they exhibit effective Th1 cell responses and are able to control Leishmania major infection. These data demonstrate that Notch signaling is a regulator of type 2 immunity.

Notch signaling regulates multiple cell fate decisions in multicellular organisms. There are four mammalian Notch receptors (Notch1–4) whose signals converge in a common pathway. Upon binding to Jagged or Delta-like (Dll) ligands on an adjacent cell, two sequential proteolytic events release the intracellular domain of Notch (ICN) allowing its translocation to the nucleus where ICN converts the helix-loop-helix transcription factor CSL/RBP-J (for CBF1/RBP-Jk in mammals, Suppressor of Hairless in Drosophila melanogaster, Lag-1 in Caenorhabditis elegans) from a transcriptional repressor to a transcriptional activator. In the nucleus, Mastermind-like (MAML) proteins (1, 2) bind the ICN–CSL/RBP-J complex and act as scaffolds to recruit coactivators, such as p300 (3). MAML is required for CSL/RBP-J-dependent Notch signaling, and inhibiting the ability of MAML proteins to recruit coactivators blocks transcriptional activation by Notch (4, 5).

Notch plays many roles in hematopoiesis and lymphopoiesis (6). Notch1 is required for T lineage commitment from multipotent hematopoietic progenitors (7, 8). Subsequently, Notch is required for efficient transit through the β-selection checkpoint (9–11) and may also regulate γδ T cell development (10, 12). Recent data propose multiple functions for Notch in peripheral T cells that include activation (13–15), tolerance induction (16), and helper T cell differentiation (10, 17, 18).

Naive CD4+ T cells differentiate into Th1 or Th2 cells when stimulated through their T cell antigen receptor. Th1 cells are characterized by expression of the transcription factor T-bet and produce IFN-γ, which regulates cell-mediated protection against intracellular microbes such as Leishmania major (19). Th2 cells are characterized by expression of GATA3 and the cytokines IL-4, IL-5, and IL-13, which mediate protection against parasitic helminths such as Trichuris muris (20).

Several studies implicate Notch signaling in CD4+ Th1 and Th2 cell differentiation. In vitro studies showed that expression of different Notch ligands on APCs have distinct effects on CD4+ T cell cytokine production. For example, Dll-1 induced IFN-γ (17, 18), whereas Jagged1 induced IL-4 (18). Individual Notch receptors may also induce different responses, as ICN3 up-regulated T-bet and IFN-γ expression (17), whereas ICN1 promoted expression of GATA3 and IL-4 (18). The involvement of Notch in Th cell development is likely to be more complex than in vitro experiments suggest, as mice that conditionally inactivate Notch1 in their mature T cells have normal immune responses (21) and Dll ligands suppress IFN-γ in some circumstances (16). Furthermore, mature T cells that are incapable of Notch signaling as a result of CSL/RBP-J deletion are impaired in IL-4 but not IFN-γ production in vitro (10, 18). In
contrast, use of a γ-secretase inhibitor, a pharmacological inhibitor of Notch signaling among other pathways, inhibited only Th1 cell differentiation (22). Thus, it has not yet been resolved whether Notch regulates Th1 cell differentiation, Th2 cell differentiation, or both.

To elucidate the role of Notch signaling in peripheral T cell function, we engineered mice to conditionally express a dominant negative MAML (DNMAML) protein that blocks recruitment of coactivators by MAML proteins, thereby preventing transcriptional activation by all four Notch receptors (4, 5). Although T cell development and activation were normal, IL-4 production was impaired. When challenged with pathogens in vivo, mice that express DNMAML developed an effective Th1 cell response against L. major, but were susceptible to T. muris infection. These data show that CSL/RBP-J–dependent Notch signaling has a specific role in promoting type 2 immunity.

RESULTS AND DISCUSSION

Generation of mice

To block Notch signaling from all four Notch receptors, mice were generated to express a GFP tagged DNMAML protein in a tissue-specific manner. DNMAML contains amino acids 13–74 of MAML1 that bind the Notch–CSL/RBP-J complex but lacks the MAML1 sequences required to recruit transcriptional coactivators (4). The targeting construct includes a loxP flanked (floxed) PGK-Neo-tPA cassette preventing transcriptional read-through of DNMAML1-GFP (DNMAML) and was knocced into the ROSA26 locus (23) (Fig. 1 A). Upon Cre expression, the PGK-Neo-tPA is excised, thereby allowing DNMAML expression and inhibition of Notch signaling. We previously showed that DNMAML blocks signaling by all four Notch receptors by reporter assays (4, 5). In addition, expression of DNMAML in BM progenitors blocks Notch1-dependent T cell development, and expression in splenocytes inhibits Notch2-dependent marginal zone B cell development (24). Furthermore, DNMAML prevents T cell development when experimentally driven by Notch3 or Notch4 (Fig. S1, available at http://www.jem.org/cgi/content/full/jem20050923/DC1). To date, we have recapitulated only Notch loss of function phenotypes with DNMAML and have not observed “off-Notch” effects. Therefore, DNMAML is a pan-Notch inhibitor, blocking signaling from all four Notch receptors.

To study Notch function in CD4+ T cells, CD4+cre transgenic mice were bred with DNMAML+/+ mice to obtain offspring that included CD4+cre × DNMAML+/+ (CCD) and CD4+cre (CC) littermate controls (Fig. 1 A). Thymocytes from CCD mice were stained with antibodies against cell surface markers for T cell subsets and further analyzed for GFP by flow cytometry (Fig. 1 B). GFP was detectable as early as the double negative 3 stage (Fig. 1 C, DN3; ∼2%) and gradually increased through DN4 and intermediate single positive stages (Fig. 1 C, ISP). GFP expression was high by the double positive (DP) stage (∼93%) and reached ∼99% in single positive (SP) thymocytes. In addition, T cell subsets were purified and further sorted on the basis of GFP. Deletion of the floxed cassette was demonstrated by PCR on genomic DNA from sorted T cell subsets, which correlated with GFP expression (Fig. 1 C). Together, these data show that CD4+cre driven expression of the DNMAML transgene occurred in CCD mice.

Normal T cell development in CCD mice

Thymic cellularity and absolute numbers within all T cell subsets were normal in CCD mice (Fig. 2 A). In addition, the percentages of CD4+ and CD8+ single positive T cells were similar in CC and CCD mice (Fig. 2 B). Likewise, absolute numbers (Fig. 2 C) and percentages of T cells were normal in the spleen (Fig. 2 D) and lymph nodes (not depicted). In the periphery, >98% of CD4+ or CD8+ T cells were GFP+, demonstrating that single positive T cells had not encountered any selective pressures upon transit from the thymus to the periphery (Fig. 2 E). Peripheral T cells from CCD mice were stained for activation markers such as CD69, CD25, CD62L, and CD44. No differences were observed in baseline expression (Fig. 2 F).

Figure 1. Generation of mice. (A) Partial restriction map of the ROSA26 locus and targeting construct. The PGK-Neo-tPA cassette is flanked by two loxP sequences (black triangles). Downstream is DNMAML1-GFP-bpA (DNMAML). Mice expressing one knocked-in allele were crossed with CD4-cre mice. CD4-cre (CC) and CD4-cre × DNMAML1 (CCD). Three arrows indicate the position of PCR primers used to detect the deletion. SA, adenovirus splice acceptor; tPA, trimer of the SV40 polyadenylation sequence; bpA, bovine growth hormone polyadenylation sequence. (B) Thymi from CCD mice were stained with antibodies delineating T cell subsets and further analyzed for GFP expression by flow cytometry. Numbers represent the percentage of GFP+ cells. DN, double negative; DP, double positive; ISP, intermediate single positive. (C) T cell subsets were sorted on the basis of GFP and PCR was done to detect the floxed allele (top band) or the allele that has undergone Cre-mediated excision (bottom band). White lines indicate that intervening lanes have been spliced out.
Loss of Notch signaling impairs Th2 cell differentiation in vitro

To characterize CD4+ T cell function in CCD mice, CD4+ T cells were stimulated in vitro with a range of concentrations of anti-CD3 and anti-CD28 and harvested at different time points. There were no differences in up-regulation of activation markers with any of the conditions compared with CC controls (Fig. 3 A). CC and CCD CD4+ T cells underwent similar rounds of division, as shown by CFSE dilution (Fig. 3 B).

To determine whether Notch influenced Th cell differentiation, purified CD4+ and CD4+GFP+ T cells from CC and CCD mice, respectively, were isolated and cultured with irradiated APCs in the presence of anti-CD3, anti-CD28, and IL-2 under neutral, Th1, or Th2 cell conditions. GFP-expressing cells were previously used as a control to show that GFP did not affect Th cell differentiation (unpublished data). After 7 d of culture, cells were restimulated with PMA and ionomycin for intracellular staining or anti-CD3 for ELISA to detect cytokine production. Intracellular staining showed a significant decrease in the percentage of cells expressing IFN-γ in CCD cultures compared with CC controls under neutral and Th2-polarizing conditions (Fig. 3 C). Although culturing under Th2 cell-permissive conditions allowed more cells to differentiate into IL-4 producers, the frequency of IL-4+CD4+ T cells from CCD mice was still less than that observed in CC cultures. CD4+ T cells also exhibited a decrease in the mean fluorescence intensity of IL-4 staining compared with CC controls (not depicted) correlating with a substantial decrease in IL-4 secretion (Fig. 3 D).

The ability of CCD cells to produce IL-4, albeit in reduced quantities, in Th2 cell conditions suggests that Notch provides an important additive signal in promoting Th2 cell differentiation, but is not an absolute requirement.

The percentage of IFN-γ-producing cells increased in neutral and Th2 cell conditions compared to CC controls (Fig. 3 C). However there was no change in the percentage of IFN-γ-producing cells in Th1 cell conditions. This was in contrast to the increase in IFN-γ detected by ELISA in all conditions and suggests that there may be differences in secretion of IFN-γ over time (Fig. 3 D). Production of IFN-γ in Th2 cell conditions may have been caused by the strong restimulation conditions. Together, these studies demonstrated that whereas CD4+ Th1 cell differentiation was intact in CCD CD4+ T cells, Notch signaling was required for optimal Th2 cell differentiation and expression of IL-4. However, it is unclear whether Notch directly promotes Th2 cell differentiation. It is possible that the absence of Notch may lead to an uncontrolled Th1 cell response that inhibits Th2 cell differentiation.

CCD mice fail to mount a Th2 cell response against *T. muris*

To investigate the in vivo requirement for Notch in response to an infection that normally elicits a protective Th2 cell re-
response, CCD mice were infected with *T. muris*, a helminth pathogen of the murine gastrointestinal tract. Immunity is dependent on CD4+ Th2 cells that secrete IL-4 and IL-13. Genetically resistant B6 mice generate a Th2 cytokine response, which promotes goblet cell responses and control of infection by ~21 d after infection (25). As expected, mesenteric lymph node cells isolated from *T. muris*-infected CC littermate controls exhibited robust parasite-specific IL-4, -5, and -13 responses (Fig. 4 A). In contrast, *T. muris*-infected CCD mice displayed decreased IL-4, -5, and -13 production, whereas IFN-γ production was normal compared with CC mice (Fig. 4 A). Consistent with a defect in the expression of Th2 cytokines, infected CCD mice showed decreased levels of *T. muris*-specific serum IgG1 but IgG2a was increased (Fig. 4 B). Furthermore, total serum IgE, another hallmark of type 2 inflammation, was dramatically lower in infected CCD mice compared with infected CC mice. Histology of the gut revealed that although infected CC mice had pronounced Th2 cytokine-dependent goblet cell and mucin responses, there was an absence of goblet cell responses in infected CCD mice at day 21 after infection (Fig. 4 E). Whereas CC mice had a low worm burden at day 21, CCD mice exhibited high worm burdens, which is indicative of a persistent infection (Fig. 4 D). The level of infection in CCD mice was equivalent to that of immunodeficient RAG2−/− mice, reflecting the severely impaired immune response in CCD mice. Furthermore, infection persisted in CCD mice at day 32 after inoculation (Fig. 4 F). Together, these results show that Notch signaling is required for optimal Th2 cytokine responses and resistance to helminth infection.

**CCD mice are resistant to *L. major* infection**

To determine whether CCD mice have a specific defect in Th2 cell differentiation or a global defect in mounting immune responses in vivo, CC and CCD mice were infected with *L. major*. After infection, genetically susceptible BALB/c mice developed progressive nonhealing lesions (Fig. 5 A). In contrast, infected CC and CCD mice efficiently controlled parasite replication (not depicted) and resolved their lesions (Fig. 5 A). To determine cytokine production, mice were killed at day 20 after infection, and popliteal LN (PLN) cells were restimulated with soluble *L. major* antigen. Consistent with resistance to infection, CCD mice produced abundant amounts of IFN-γ (Fig. 5 B). *L. major*-infected CCD mice produced undetectable levels of IL-4 compared with low, but detectable, levels of IL-4 in PLN cultures isolated from infected CC mice (Fig. 5 C). Thus, in the absence of Notch signaling Th1-mediated immunity is preserved.

In summary, pan inhibition of Notch signaling in murine T cells reveals a critical requirement for Notch in efficient Th2 cell generation, both in vivo and in vitro. In the ab-

**Figure 4.** CCD mice fail to mount a Th2 cell response against *T. muris*. (A) At day 21, MLN cells from *T. muris* infected CC and CCD mice were stimulated with *T. muris* antigen for 72 h, and cytokine levels were detected by ELISA. (B) *T. muris*-specific IgG1 and IgG2a and total IgE (C) from day 21-infected mice were determined by ELISA. (D) Worm burden at day 21 after infection. (E) Gut sections stained for intracellular mucin to detect goblet cells. (F) Worm burden at day 32 after infection. Data are representative of three independent experiments with at least nine mice in each group. Data show the mean ± 2 SEM; *, P < 0.05; **, P < 0.01.

**Figure 5.** CCD mice are resistant to *L. major* infection. (A) Lesion size of footpads infected with *L. major*. (B) PLN cells from *L. major* infected mice were restimulated with soluble *Leishmania* antigen for 4 d and intracellular IFN-γ expression was detected by flow cytometry. Cells are gated on CD4+ T cells. Numbers represent the percentage of cells within the gates. (C) Supernatants were collected and levels of IL-4 were detected by ELISA. Data show the mean ± SD (n = 4).
sence of Notch, mice were impaired in the development of a Th2 cell response and failed to clear a parasitic infection. Furthermore, there was no evidence of global immunodeficiency, and mice were able to mount a normal Th1 cell response and control replication of an intracellular pathogen.

The failure to develop a sufficient Th2 cell response in CCD mice shows that optimal Th2 cell immunity is dependent on nuclear Notch signaling through CSL/RBP-J. Potential molecular targets of Notch signaling in Th2 cell differentiation include GATA-3 and IL-4. Both contain conserved CSL/RBP-J binding sites in important regulatory regions and the IL-4 regulatory region responded to Notch signals in vitro (18). Alternatively, Notch may promote Th2 cell differentiation by suppressing Th1 cell differentiation.

The normal Th1 cell response in CCD mice is strong evidence that Th1 cell differentiation does not require Notch signaling through the ICN–MAML–CSL/RBP-J transcriptional activation complex. This is difficult to reconcile with overexpression data showing that ICN potentiates IFN-γ production and can bind to the T-bet promoter (17, 22). Our data do not rule out effects of Notch signaling that are independent of CSL/RBP-J or MAML (26), however, this pathway is poorly understood.

Although our results do not exclude distinct roles for Notch ligands and receptors in Th1 and Th2 cell differentiation, they provide a foundation for determining the precise molecular pathways by which Notch controls helper T cell differentiation. Furthermore, our data suggest that Notch signaling is a potential therapeutic target in diseases characterized by pathologic Th2 cytokine responses, such as asthma, autoimmunity, and forms of inflammatory bowel disease.

MATERIALS AND METHODS
Generation of ROSA26 DNAML1-GFP mice. The gene-targeting vector was constructed using a ROSA26 targeting vector generated by Smivas et al. (23). DNAML1 (aa 13–74) was fused with GFP (DNAML1-GFP) (4). To generate the ROSA26–DNAML1-GFP targeting construct, the KpnI site in the linker between DNAML1 and GFP was deleted by PCR-directed mutagenesis to replace GGTACC with GGCATT. Both GGT and GGC encode the amino acid glycine. DNAML1-GFP was digested with BglII, Klenow filled, and then digested with NotI to excise the DNAML1–GFP cDNA. The resulting fragment was inserted into pBlgT (23) digested with SalI, Klenow filled, and then digested with NotI. pBlgT was digested with PacI and Ascl to release the entire floxed neo-tpA and DNAML1-GFP assembly and inserted into pROSA26PA (23) digested with SalI, Klenow filled, and then digested with NotI. pBigT was digested with PacI and AscI. This plasmid was subsequently linearized by digestion with KpnI and electroporated into embryonic stem cells. Approximately 180 G418- and diphtheria toxin-resistant clones were first screened by PCR using two primers—ROSA26 flanking (5′ CCT AAG GAA GAG CCT GTG CTT TGG 3′) and splice acceptor (5′ CAT CAA GGA AACC GTA CTA CTG 3′). The size of the PCR product was ~1.2 kb (27). PCR amplification was performed as follows: 93°C for 1.5 min, and then 40 cycles of 93°C for 30 s, 57°C for 30 s, followed by 65°C for 3 min. Next, 23 appropriately targeted clones were verified by Southern blot analysis using EcoRV digestion and hybridized with a probe recognizing the ROSA26 genomic sequences but not the targeting vector intended for homologous recombination. An 11-kb fragment from the wild-type allele and a new 3.8-kb fragment from the targeted allele were expected. Subsequently, the targeted embryonic stem cell clones were microinjected into C57BL/6 (B6) blastocysts by either the Abramson Family Cancer Research Institute Transgenic Mouse Core or the University of Pennsylvania Molecular Cardiology Research Center Transgenic Core. Chimeric males were bred with B6 females and heterozygous offspring were screened by PCR and Southern blot of tail DNA. These mice were designated as DNAML1+. To express DNAML1-GFP in mature Th cells, DNAML1+ mice were crossed with CD4-Cre transgenic mice (Taconic Farms). Mice expressing one allele of DNAML1 were designated as CCD, whereas mice expressing only the CD4-Cre transgene were designated as CC. Mice were backcrossed onto the B6 background for at least five generations. CCD mice were compared with littermate CC controls in all experiments.

B6 and RAG2−/− mice were obtained from Taconic Farms. Experiments were performed according to guidelines from the National Institutes of Health and with an approved protocol from the University of Pennsylvania Animal Care and Use Committee.

In vitro T cell differentiation. CD4+CD25− T cells from CC or CCD mice were sorted and cultured in IMDM (Life Technologies) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 2 mM l-glutamine, and 50 μM 2-mercaptoethanol. T cells were stimulated with irradiated APCs and 0.1 μg/ml anti-CD3, 0.5 μg/ml anti-CD28, and 10 U/ml of recombinant human IL-2. No additional antibodies or cytokines were added to nonpolarizing conditions. 10 μg/ml anti–IL-4 and 5 ng/ml of recombinant mIL-12 (Peprotech) was added in Th1 cell conditions. 10 μg/ml anti–IL-12 and 20 ng/ml of recombinant mIL-4 (Peprotech) was added in Th2 cell conditions. Cells were differentiated for 7 d and restimulated with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) for 4 h. During the last 2 h, 2 μg/ml Brefeldin A (Sigma-Aldrich) was added. Cells were washed, fixed in 4% paraformaldehyde and permeabilized in 2% saponin (Sigma-Aldrich). For ELISAs, equal numbers of viable CD4+ T cells were plated in triplicate and restimulated with 2.5 μg/ml of plate-bound anti-CD3. After 24 h, supernatants were collected and standard sandwich ELISA protocols were used. CFSE labeling was performed as previously described (14).

Flow cytometry, cell sorting, and antibodies. After incubating with 2.4G2 hybridoma supernatant to block Fc receptors, cell suspensions were stained with primary antibodies and washed in 2% PBS and 0.01% NaN3. Data were acquired on FACSCalibur (Becton Dickinson) and analyzed using FlowJo software (Tree Star). CD4+ and CD4+GFP+ T cells were sorted on a MoFlo cytometer (DakoCytomation). All antibodies were obtained from BD PharMingen except anti–IL-13 antibodies, which were from R&D Systems.

T. muris infection and antibodies. T. muris was maintained in genetically susceptible or immunocompromised mice. Between days 35 and 42 after infection, adult worms were isolated and cultured in RPMi 1640 (Life Technologies) containing 500 μg/ml penicillin and 500 μg/ml streptomycin for 24 h. T. muris excretory–secretory antigen was isolated at 4 and 24 h, dialyzed, and sterile filtered, and protein concentrations were determined by Bradford assay. Antibody preparations were used in lymphocyte restimulations (50 μg/ml). Deposed eggs were collected after 24 h of culture, washed three times in sterile water and incubated at room temperature for 6 wk and stored at 4°C. Mice were infected on day 0 with 150–200 embryonated eggs, and parasite burdens were assessed on day 21 after infection. Mesenteric LN (MLN) cell suspensions were prepared and reexpanded in IMDM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. Cells were plated at 4 × 105 cells/well and cultured alone or in the presence of T. muris Ag for 72 h. Levels of IL-4, -5, and -13 and IFN-γ were assayed by sandwich ELISA. For histology, segments of mid-cecum were removed, washed in sterile PBS, and fixed for 24 h in paraformaldehyde. Tissues were processed and paraffin embedded using standard histological techniques. For detection of intestinal goblet cells, 5-μm sections were cut and stained with hematoxylin and eosin or Alcian blue periodic acid Schiff. Analysis of paraffin-embedded sections was performed by antigen capture
ELISA using biotinylated rat anti–mouse IgG1 and IgG2a (BD Biosciences). Total serum levels of IgE were determined by ELISA (BD Biosciences).

**L. major infection and antigen.** *L. major* parasites (MHOM/IL/80/Friedlin) were grown in Grace’s insect culture medium (Life Technologies) supplemented with 20% heat-inactivated FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. Soluble Leishmania Ag (SLA) was prepared as previously described (28). Mice were injected in the hind left footpad with \( 5 \times 10^4 \) stationary phase promastigote parasites. Footpad swelling was measured weekly using digital calipers (Mitutoyo), and lesion size was determined by subtracting the size of the uninfected contralateral footpad from the size of the infected footpad. PLN cells from infected mice were restimulated with SLA for 4 d. Intracellular staining and ELISA were performed as described above.

**Online supplemental material.** Fig. S1 shows that DNMAML can inhibit Notch3 and Notch4 driven T cell development and is available at http://www.jem.org/cgi/content/full/jem20050923/DC1.

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