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Notch Signaling Regulates Follicular Helper T Cell Differentiation

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Follicular helper T (T_{FH}) cells are specialized in providing help for B cell differentiation and Ab secretion. Several positive and negative regulators of T_{FH} cell differentiation have been described but their control is not fully understood. In this study, we show that Notch signaling in T cells is a major player in the development and function of T_{FH} cells. T cell–specific gene ablation of Notch1 and Notch2 impaired differentiation of T_{FH} cells in draining lymph nodes of mice immunized with T-dependent Ags or infected with parasites. Impaired T_{FH} cell differentiation correlated with deficient germinal center development and the absence of high-affinity Abs. The impact of loss of Notch on T_{FH} cell differentiation was largely independent of its effect on IL-4. These results show a previously unknown role for Notch in the regulation of T_{FH} cell differentiation and function with implications for the control of this T cell population. ***The Journal of Immunology*****, 2013, 191: 2344–2350.**

Follicular helper T (T_{FH}) cells play a critical role in providing help for B cells to enhance germinal center (GC) formation, the generation of high-affinity Abs, and to mature into memory B cells and long-lived plasma cells (1, 2). The plasticity of different Th cell subsets has been recently shown (3), and it remains unclear whether T_{FH} cells are a specific lineage. Following parasite infection, T_{FH} cells produce most IL-4 or IFN-γ detected in draining lymph nodes (dLNs) (4–6). Although cytokines contribute to Th cell differentiation, they can be generated in the absence of cytokine signaling, demonstrating a role for other molecules in this process (reviewed in Ref. 7).

Notch proteins are a family of evolutionary conserved transmembrane-bound receptors, which play crucial roles in binary cell fate decisions in many developmental systems. In mammals, four Notch receptors (Notch1–4) are activated by five transmembrane-bound ligands (Jagged 1 and 2 and Delta-like 1, 3, and 4). Interaction of Notch receptors with their ligands initiates the release of the active intracellular domain of Notch by proteolytic cleavages from the membrane, allowing its translocation into the nucleus. Once there, the intracellular domain of Notch forms a complex with recombination signal-binding protein-J, converting it from a repressor to an activator of transcription (8). There is increasing evidence revealing an important role for Notch signaling in the regulation of $CD4^+$ Th cell differentiation or function in the periphery (9–11). However, the role of Notch in the differentiation of T_{FH} cells has not previously been investigated.

In this study, we used mice carrying a T cell–specific deletion of Notch1 and Notch2 to show that Notch signaling in $CD4^+$ T cells is an essential component for the differentiation of T_{FH} cells. Impaired T_{FH} cell development in the absence of Notch signaling correlated with strongly reduced numbers of GC B cells and decreased generation of high-affinity Abs during T-dependent immune responses. Collectively, our data reveal a Notch-specific contribution to T_{FH} cell differentiation.

Materials and Methods

Mice

$N1N2^{lox/lox}$ CD4-*Cre* mice (referred to hereafter as $N1N2^{-/-}$) were previously described (12). $N1N2^{lox/lox}$ littermates were used as controls. All mice are on a C57BL/6 genetic background. Mice are from Charles River Laboratories (CD45.1), Harlan Olac (Bicester, U.K.; BALB/c), The Jackson Laboratory (Bar Harbor, ME; IL-$4^{-/-}$), and Taconic (IL-4Rα$^{-/-}$). CD4-*Cre* mice were also used as controls and gave similar results to $N1N2^{lox/lox}$ mice (data not shown). All mice were bred and maintained under pathogen-free conditions in the Animal Facility at the Center of Immunity and Infection Lausanne (Epalinges, Switzerland). All experimental procedures with mice were approved by the Veterinary Office Regulations of the State of Vaud, Switzerland (authorization nos. 1266-3 and 1266-4).

Parasites and immunizations

Mice were infected s.c. with 3×10^6 *Leishmania mexicana* (MYNC/BZ/62/M379) amastigotes into the back rump or were immunized with 5000 *Schistosoma mansoni* eggs injected s.c. in the hind footpad. Mice were immunized s.c. with 25 μg per site 4-hydroxy-3-nitrophenyl acetyl (NP) conjugated to chicken γ-globulin (CGG) (NP_{39}-CGG; Biosearch Technologies) in Montanide adjuvant ISA25 (25% in PBS; Seppic). dLN cells

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Abbreviations used in this article: BM, bone marrow; BTLA, B and T lymphocyte attenuator; dLN, draining lymph node; CGG, chicken γ-globulin; GC, germinal center; NP, 4-hydroxy-3-nitrophenyl acetyl; PD-1, programmed death-1; PP, Peyer's patch; T_{FH} cell, follicular helper T cell; WT, wild-type.

from *L. mexicana*–infected mice were cultured with and without UV-irradiated parasites for 72 h. The cytokine contents of the cell supernatants were measured by ELISA. IL-4 and IL-5 cytokines were analyzed with OptEIA kits (BD Biosciences), and IL-13 was analyzed with DuoSet kit (R&D Systems).

Serum Ab quantification

Ag-specific IgG1 and IgM Abs were quantified in sera by Ag-capture ELISAs and detected with biotinylated goat anti-mouse IgG1 and goat anti-mouse IgM (both from Caltag Laboratories). Total or high-affinity NP-specific IgG1 and IgM Ab levels were measured by ELISA using NP_{23}-BSA or NP_4-BSA (Biosearch Technologies).

cDNA preparation and real-time PCR

Total mRNA was extracted with the RNeasy mini kit (Qiagen), and cDNA was prepared as previously described (12). Quantitative real-time PCRs were done using SYBR Green and a LightCycler system (Roche). Primers used were previously described (4, 13, 14).

Flow cytometry

The following mAb conjugates were used to stain cells: CD4-PE-Cy5, -Alexa 700, -allophycocyanin-Alexa 750; CD62L-PE; CD44-allophycocyanin-Cy7; CD25-Pacific Blue; TCRβ-PE-Cy5.5; CD8-FITC, -Pacific Blue, -allophycocyanin; B220-FITC, -PE-Texas Red, -allophycocyanin, -PerCP-Cy5.5; programmed death-1 (PD-1)-PE; GL7-FITC; Fas-biotin; ICOS-PE-Cy5; B and T lymphocyte attenuator (BTLA)-biotin; Lineage-allophycocyanin-Alexa 750, -Alexa 700 (Gr-1, Ter119, and CD11b); CD45.1-PE-Cy5.5; CD45.2-Pacific Blue; Bcl6-Alexa 647; Foxp3-PE-Cy5. A purified anti-CXCR5 mAb followed by a goat anti-rat IgG-FITC (Caltag Laboratories, Invitrogen) was used to stain CXCR5. Biotinylated Abs were revealed with allophycocyanin-streptavidin conjugates or PE-Cy7-streptavidin. All conjugates were purchased from eBioscience, except for CXCR5, B220-PE-Texas Red, and Bcl6-Alexa 647, which came from BD Pharmingen. For T cell proliferation, dLN cells were isolated 9 d after NP-CGG immunization and stained with CFSE (Molecular Probes). Cells were then restimulated with and without 300 μg/ml NP-CGG soluble Ags for 96 h and analyzed by FACS. T cell apoptosis was analyzed by DAPI (Sigma-Aldrich) and annexin V (BioLegend) staining of dLN cells of NP-CGG–immunized mice restimulated for 72 h with *S. mansoni* soluble egg Ags. Analyses were performed on FACScan, FACSCalibur, FACSCanto, or FACS LSR II flow cytometers (Becton Dickinson) and data were processed with FlowJo (Tree Star).

Immunohistochemistry

Popliteal or inguinal lymph nodes from naive mice, or from mice infected with *L. mexicana*, injected with *S. mansoni* eggs or with NP_{39}-CGG were isolated and frozen in optimum cutting temperature embedding compound (Sakura Finetek). Sections of 7 μm were cut with a Leica cryostat and fixed in acetone. Sections were blocked with normal mouse and donkey serum and then stained with the following primary Abs: peanut agglutinin-biotin (Vector Laboratories), rat anti-B220 (RA3-6B2), rat anti-CD4 (H129.19.6), sheep anti-IgD (The Binding Site), rabbit anti–Lyve-1 (ReliaTech), Syrian hamster anti-gp38 (clone 8.1.1), rat anti-CD31 (clone GC51), Armenian hamster anti-CD11c (clone N418), and rat anti-syndecan biotin (BD Pharmingen). Secondary Abs used were: donkey anti–rat-Cy3, -biotin, or -Alexa 488; donkey anti–sheep-allophycocyanin or -Alexa 647; donkey anti–rabbit-Alexa 488, -streptavidin-Cy3, or -HRP; goat anti-Armenian hamster-biotin (all from Jackson ImmunoResearch Laboratories) or -streptavidin-Alexa 488 (Invitrogen). All images were captured with an Axiovert 200M microscope and analyzed using Adobe Photoshop.

Mixed bone marrow chimeras

CD45.1 mice were injected i.p. with 100 μg purified anti-NK1.1 Ab (clone PK136). One day later, mice were lethally irradiated with a single dose of 900 rads. Donor bone marrow (BM) cells were isolated and incubated with anti-Thy1 culture supernatant (clone AT38), DNase1, and rabbit complement (Saxon Rabbit Company). A total of 1×10^7 donor cells were engrafted i.v. into irradiated CD45.1 mice at a ratio of CD45.1/control or $N1N2^{-/-}$ of 1:3. Chimerism was analyzed after 8 wk of engraftment by FACS analysis.

Statistical analysis

Data were analyzed using the Student *t* test for unpaired data. When more than two variables were tested, the statistical analysis was performed using a one-way ANOVA followed by a Tukey multiple comparison test.

Results

Notch expression on T cells is essential for the differentiation of T_{FH} cells

To evaluate whether the absence of Notch expression on T cells affected T_{FH} cell differentiation, mice with deletion of both Notch1 and Notch2 specifically in the T cell lineage ($N1N2^{-/-}$) were used, because these two receptors were shown to be selectively induced on activated peripheral $CD4^+$ T cells (15). The frequency and number of $CD4^+$ T cells (Fig. 1A) including naive $CD4^+$ cells ($CD62L^+CD44^{low}$) and $CD25^+Foxp3^+$ natural regulatory T cells were normal in $N1N2^{-/-}$ mice, comparable to those of control mice (Fig. 1B). The population of $CD8^+$ T cells was normal as well (Fig. 1C). Of note, the lymph node architecture, with distinct T and B zones as well as the distribution of different hematopoietic and stromal cell populations in naive $N1N2^{-/-}$ mice was comparable to that of control mice (Supplemental Fig. 1). Following infection with *L. mexicana* or injection of *Schistosoma mansoni* eggs, the total number of cells as well as the number of $CD4^+$ T cells in the dLNs were similar in $N1N2^{-/-}$ and control mice (Fig. 1D). Additionally, upon Ag stimulation, $CD4^+$ T cell proliferation did not differ between both groups. No difference in T cell apoptosis was observed in $N1N2^{-/-}$ and control Ag–stimulated dLN $CD4^+$ T cells (Supplemental Fig. 2).

Having established that $N1N2^{-/-}$ mice do not have defects in peripheral T cell populations, these and control mice were immunized with the well-defined T cell–dependent Ag NP conjugated to CGG in Montanide adjuvant. $CD4^+$ T_{FH} cells were identified in dLNs of immunized mice by FACS through their unique combined high expression of CXCR5, PD-1, BTLA, and the presence of the transcription factor Bcl6. Seven days after immunization, the percentage of T_{FH} cells in dLNs of immunized $N1N2^{-/-}$ mice was significantly lower than in control mice. $N1N2^{-/-}$ T_{FH} cells expressed reduced levels of the classical T_{FH} cell markers CXCR5, PD-1, BTLA, and Bcl6, whereas they expressed normal levels of ICOS (Fig. 2A). To assess whether this decreased frequency of T_{FH} cells was sustained following immunization, the kinetics of T_{FH} cell development were measured in $N1N2^{-/-}$ and control mice following NP-CGG immunization. At all time points, the frequency of T_{FH} cells was markedly reduced in $N1N2^{-/-}$ mice compared with control mice (Fig. 2B) whereas the number of T_{FH} cells was significantly reduced from 7 d on (Fig. 2C).

To investigate whether Notch receptor signaling also contributes to T_{FH} cell differentiation during physiological immune responses, $N1N2^{-/-}$ and control mice were inoculated with *L. mexicana* amastigotes or with *S. mansoni* eggs, both parasites or parasite products inducing the differentiation of T_{FH} cells. Indeed, infection with *L. mexicana* or inoculation of *S. mansoni* eggs led to the differentiation of T_{FH} cells in control mice. In contrast, very few T_{FH} cells were detectable in $N1N2^{-/-}$ mice (Fig. 3A). Both the frequency and total number of T_{FH} cells in dLNs of infected $N1N2^{-/-}$ mice were strongly reduced (Fig. 3B). Of note, activation of $N1N2^{-/-}$ non–T_{FH} cells was not impaired, as shown by the presence of PD-$1^{int}CD4^+$ T cells. To visualize the presence of T_{FH} cells in GCs, $CD4^+$ T cell immunohistology was performed in dLNs of *L. mexicana*–infected mice. Numerous $CD4^+$ T cells were clearly visible in the GCs of $N1N2^{lox/lox}$ control mice, whereas only a few scattered $CD4^+$ T cells were detectable over the primary B cell follicles of $N1N2^{-/-}$ dLNs (Fig. 3C). Collectively, these data demonstrate that Notch receptors on T cells play a crucial role in the development of T_{FH} cells during immune responses.

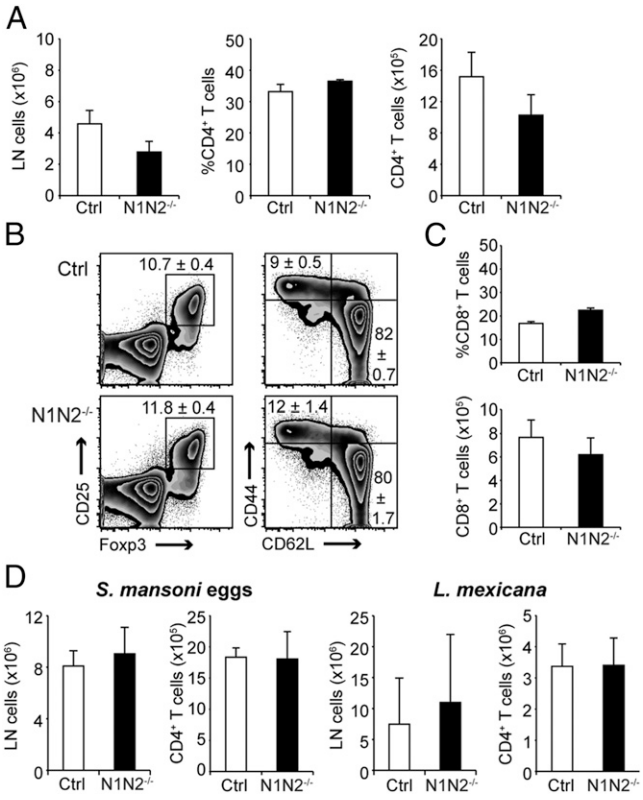

FIGURE 1. Normal $CD4^+$ and $CD8^+$ homeostasis in naive $N1N2^{-/-}$ mice. Inguinal lymph nodes of naive $N1N2^{-/-}$ and control (Ctrl) mice were analyzed by FACS. (**A**) The total cell number in dLNs and the frequency and number of $CD4^+$ T cells are given. Error bars represent SEM for $n = 3$ mice/group. (**B**) Representative FACS plots, pregated on $CD4^+$ T cells, display the frequency of $CD25^+Foxp3^+$ natural regulatory T cells and the activation status of $CD4^+$ T cells. Numbers in quadrants represent the frequency of the gated population ± SEM of $n = 3$ mice/group. (**C**) The percentage and number of $CD8^+$ T cells are shown. Error bars represent SEM for $n = 3$ mice/group. (**D**) The total cell number in dLNs and of $CD4^+$ T cells in dLNs upon *S. mansoni* eggs immunization or *L. mexicana* infection is given. Error bars represent SEM for $n = 3$ mice/group. Data are representative of two independent experiments.

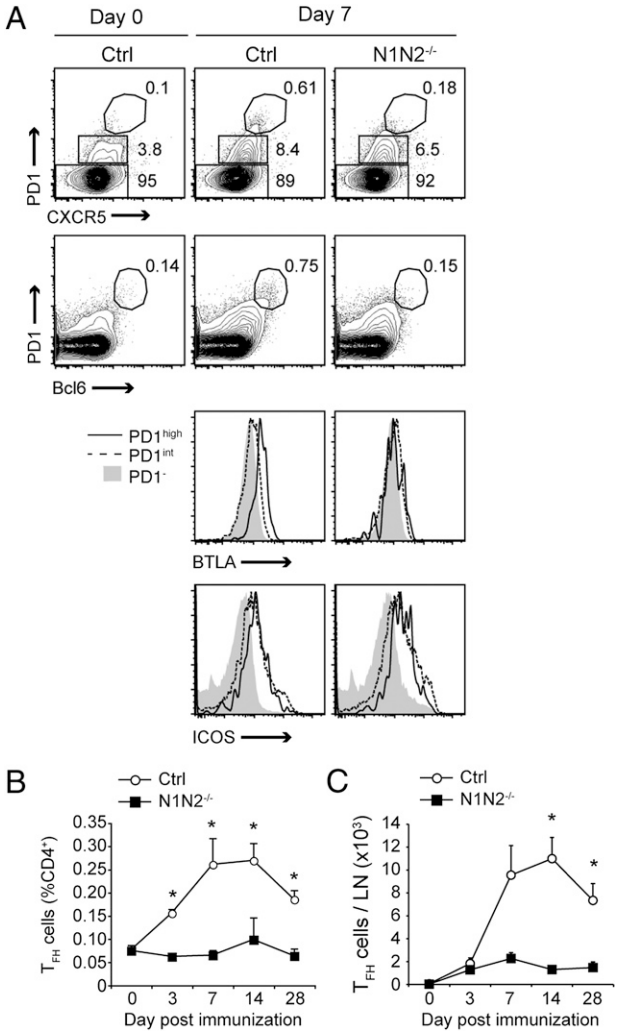

FIGURE 2. Notch is essential in T_{FH} cell differentiation following NP-CGG immunization. $N1N2^{-/-}$ and control mice were immunized or not (day 0) with NP-CGG. (**A**) Representative FACS plots show the T_{FH} cell population according to their expression of PD-1, CXCR5, or Bcl6. Histograms show the expression of BTLA and ICOS in PD-1^-, PD-1^{int}, and T_{FH} cells as gated on the *top* line. Numbers represent the frequency of the gated population. (**B** and **C**) The (B) frequency and (C) number of T_{FH} cells (PD-$1^{high}$$CXCR5^+$) gated on $CD4^+$ T cells were measured by FACS at different time points after immunization. Error bars represent the SEM for $n \geq 5$ mice/group. Data are representative of two or more distinct experiments. $^*p < 0.05$.

GC formation is impaired in immunized or infected Notch-deficient mice

Because T_{FH} cells contribute to GC development and maintenance (1), we next compared GC B cells in NP-CGG–immunized $N1N2^{-/-}$ and control mice. Remarkably, relative to control mice, the number of GC B cells ($Fas^+GL\text{-}7^+$) was markedly decreased in $N1N2^{-/-}$ mice (Fig. 4A). Similarly, *L. mexicana*–infected or *S. mansoni* eggs–exposed $N1N2^{-/-}$ mice showed a strong reduction in the frequency of GC B cells (Fig. 4B). No detectable GCs were visible by immunohistology in dLNs of mice immunized with NP-CGG, with *S. mansoni* eggs or in *L. mexicana*–infected mice (Fig. 4C and data not shown).

T_{FH} cells are essential to provide help to B cells for the production of high-affinity, class-switched Abs. The kinetics of NP-specific IgG1 secretion were quantified in the sera of NP-CGG–immunized mice, measuring high-affinity (NP_4) and total (high and low) affinity (NP_{23}) Abs. From 7 d on, with a maximum at 28 d after immunization, high-affinity serum IgG1 Abs were observed in control mice. In contrast, in line with the reduced number of GCs, hardly any high-affinity IgG1 Abs were detectable in sera from $N1N2^{-/-}$ immunized mice, with a statistically significant decrease visible 14 and 28 d after immunization (Fig. 4D). The absence of Notch receptor expression on T cells also affected the secretion of total NP-specific IgG1 Abs 28 d after immunization, consistent with the low number of GCs in these mice (Fig. 4D). NP-specific IgM Ab levels were not statistically different in immunized $N1N2^{-/-}$ and control mice. These data demonstrate a crucial role for Notch expression on T cells for GC-driven generation of high-affinity Abs following immunization with T-dependent Ags.

Notch affects T_{FH} cell differentiation in a cell-autonomous manner independently of its effect on IL-4

$N1N2^{-/-}$ mice have a T cell–specific loss of function for Notch1 and Notch2, implying that impaired T_{FH} cell differentiation in these mice is T cell autonomous. However, in contrast to the virtual absence of T_{FH} cells detected in immunized or infected $N1N2^{-/-}$ mice, a small number of T_{FH} cells were detectable in Peyer's patches (PPs) of naive $N1N2^{-/-}$ mice (Fig. 5A). The high frequencies of T_{FH} cells observed in PPs of naive wild-type (WT)

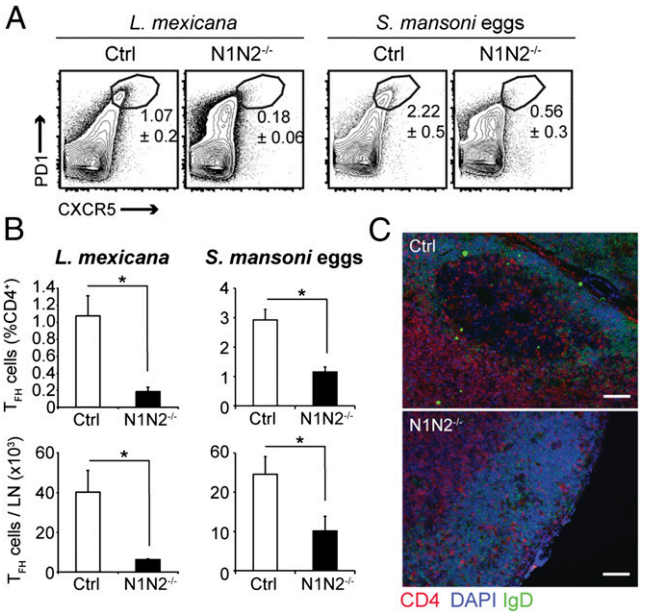

FIGURE 3. Notch is important for T_{FH} cell development under physiological conditions. N1N2$^{-/-}$ and control mice were infected with 3×10^6 *L. mexicana* amastigotes for 6 wk or immunized with *S. mansoni* eggs for 14 d. (**A**) Representative contour plots display the mean frequency of T_{FH} cells within CD4$^+$ T cells ± SEM of $n \geq 3$ mice/group. (**B**) The mean frequency and number of T_{FH} cells are given; error bars represent SEM of $n \geq 3$ mice/group. (**C**) dLN sections of *L. mexicana*–infected N1N2$^{-/-}$ and control mice were stained for CD4 (CD4$^+$ T cells), IgD (follicular mantle B cells), and DAPI. Scale bars, 50 μm. Images are representative of three independent experiments. Data are representative of at least two distinct experiments. *$p < 0.05$.

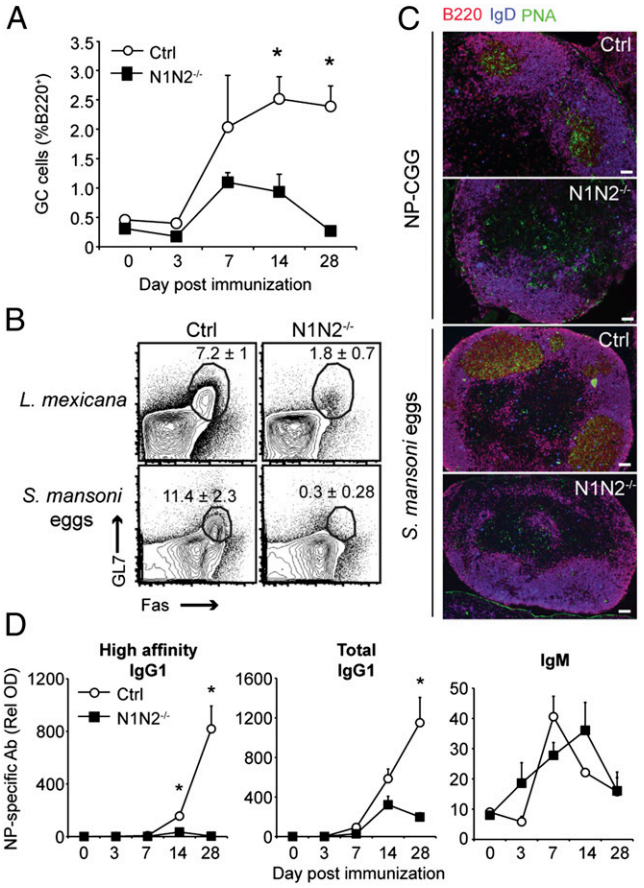

FIGURE 4. The absence of Notch on T cells affects GC B cell development. (**A**) dLNs of NP-CGG–immunized N1N2$^{-/-}$ and control mice were analyzed for the percentage of PD-1$^+$GL7$^+$ GC B cells by FACS, pregating on the B220$^+$ population. Error bars represent SEM for $n \geq 5$ mice/group. (**B**) N1N2$^{-/-}$ and control mice were inoculated s.c. with *L. mexicana* or *S. mansoni* eggs. Representative FACS contour plots display the frequency of GC B cells ± SEM for $n \geq 3$ mice/group. (**C**) dLN sections of NP-CGG– or *S. mansoni* egg–immunized N1N2$^{-/-}$ and control mice were stained for B220 (B cells), PNA (GC B cells), and IgD (follicular mantle B cells). Scale bar, 50 μm. Images are representative of three independent experiments. (**D**) High (NP_4 binding) and total (NP_{23} binding) affinity NP-specific IgG1 and total IgM Ab levels were measured by ELISA in the sera of NP-CGG–immunized N1N2$^{-/-}$ and control mice. Error bars represent the SEM for $n \geq 5$ mice/group. Data are representative of two or more distinct experiments. *$p < 0.05$.

mice correlate with the very high activity of their GCs necessary to regulate the gut microbiota (16). The small proportion of T_{FH} cells observed in N1N2$^{-/-}$ mice suggests that in the presence of sustained Ag stimulation and GCs, a small proportion of T_{FH} cells may develop independently of Notch signaling. To verify this, we generated mixed BM chimeras. Lethally irradiated CD45.1 WT mice were reconstituted with either a 1:3 mix of CD45.1 WT/CD45.2 N1N2$^{-/-}$ BM or, as a control, of CD45.1 WT/CD45.2 N1N2$^{lox/lox}$ control BM. Eight weeks later, the chimeras were immunized with NP-CGG and the relative numbers of T_{FH} and GC B cells derived from the CD45.2 (N1N2$^{-/-}$ or control) or CD45.1 (WT) donor marrow were determined. The CD45.2/CD45.1 T_{FH} cell ratio was significantly decreased in the mixed WT/N1N2$^{-/-}$ BM chimeras compared with that of control WT/N1N2$^{lox/lox}$ chimeras. In contrast, the CD45.2/CD45.1 ratio of non-T_{FH} cells was similar (Fig. 5B, 5C). The number of GC B cells derived from CD45.2 control or N1N2$^{-/-}$ BM was comparable in the two types of mixed BM chimeras (Fig. 5D), and similar levels of NP_4 high-affinity or NP_{23} high- and low-affinity Abs were detected in sera of both types of mixed chimeras (data not shown). Collectively, these data demonstrate that the impaired T_{FH} cell development in the absence of Notch is mainly T cell–intrinsic. Additionally, these data show that in the presence of WT T_{FH} cells, GC B cells can develop normally in N1N2$^{-/-}$ mice.

Several studies showed that following infection with Th2-inducing parasites, most cells secreting IL-4 in dLNs were T_{FH} cells (4–6). The direct effect of Notch on IL-4 transcription (17, 18) is distinct from that on the differentiation of T_{FH} cells. Indeed, T_{FH} cells can develop in the absence of IL-4 (4, 6) (Supplemental Fig. 3A, 3B), showing that the deficient development of T_{FH} cells in N1N2$^{-/-}$ mice is not an indirect consequence of decreased IL-4 production.

Nonresponsiveness to IL-4 has been associated with the development of smaller GCs (6, 19). Accordingly, a decreased percentage of GC B cells was measured in the dLNs following NP-CGG injection in IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice. However, the frequency of GC B cells measured in immunized IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice remained significantly higher than that measured in dLNs of N1N2$^{-/-}$ mice, whereas no difference was observed between WT BALB/c and control C57BL/6 mice (Supplemental Fig. 3C–E).

Collectively, these data reveal that Notch signaling has a critical T cell–intrinsic effect on T_{FH} cell development, which is distinct from its direct effect on IL-4.

Notch affects the balance between Bcl6 and Blimp1 mRNA levels during the differentiation of Th subsets

Thus far our data reveal a crucial role of Notch in T_{FH} cell differentiation. The transcription factor Bcl6 is the master regulator of T_{FH} cells, whereas Blimp1 is an antagonist of T_{FH} cell differ-

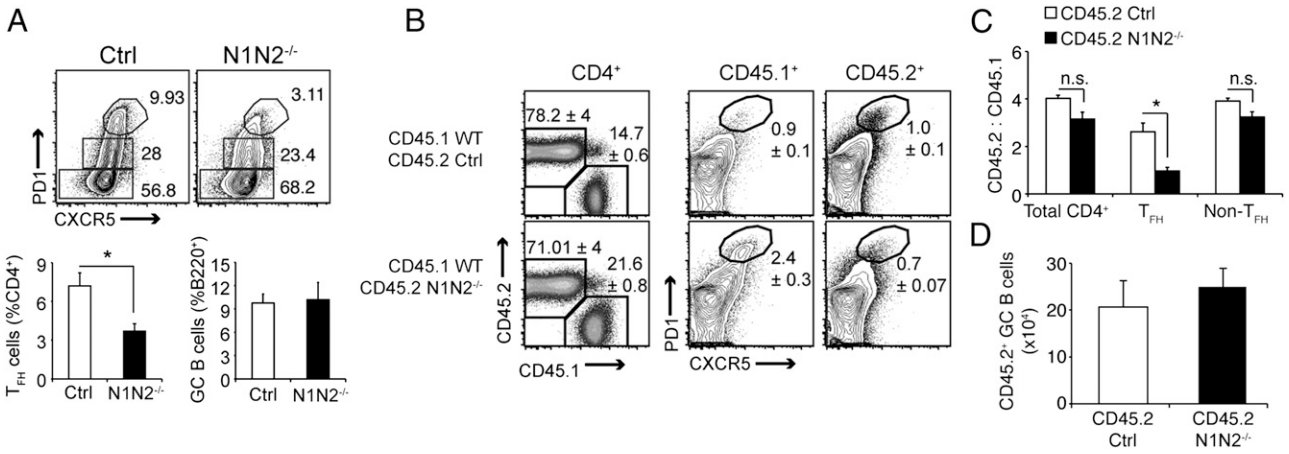

FIGURE 5. Notch affects T_{FH} cell differentiation in a cell-autonomous manner independently of its effect on IL-4. (**A**) The T_{FH} and GC B cell populations were analyzed by FACS in PPs of naive $N1N2^{-/-}$ and control mice. Representative FACS plots display the mean frequency of T_{FH} cells ± SEM of $n \geq 5$ mice/group. Histograms show the mean frequency of T_{FH} cells and GC B cells ± SEM of $n \geq 5$ mice/group. (**B**–**D**) CD45.1 WT mice were lethally irradiated and reconstituted with a 1:3 ratio of WT CD45.1 to control or $N1N2^{-/-}$ CD45.2 BM donor cells. Eight weeks later mice were immunized with NP-CGG and dLNs were analyzed for (B, C) the CD45.1/CD45.2 ratio in total $CD4^+$ T cells, T_{FH}, and non–T_{FH} cells and (D) the number of $CD45.2^+$ GC B cells. Error bars represent SEM. All data are representative of two experiments including five to six mice per group. $*p < 0.05$.

entiation (20, 21). To further investigate the mechanism of Notch action in T_{FH} cell differentiation, $CD4^+CXCR5^-PD\text{-}1^-$ (nonactivated, non-T_{FH}), $CD4^+CXCR5^-PD\text{-}1^{int}$ (pre-T_{FH}), and $CD4^+CXCR5^{high}PD\text{-}1^{high}$ (T_{FH}) cells from $N1N2^{-/-}$ and control mice were FACS sorted 7 d after immunization with NP-CGG (Fig. 6A). As expected, a marked increase in Bcl6 mRNA was measured in immunized control T_{FH} cells and not in pre–T_{FH} cells. In contrast, only the low Bcl6 mRNA level was induced in $N1N2^{-/-}$ T_{FH} cells, in line with the very low number of these cells found in dLNs of $N1N2^{-/-}$ mice (Fig. 6B). All activated $CD4^+$ T cells showed a significant increase in Blimp1 mRNA, with a markedly higher Blimp1 mRNA level in $N1N2^{-/-}$ activated pre–T_{FH} cells (Fig. 6B).

The increased Blimp1 mRNA expression in this T cell population correlated with higher levels of IL-13 and IL-5 secretion by dLN T cells of immunized or infected $N1N2^{-/-}$ mice (data not shown and Supplemental Fig. 2C). The lower levels of secreted IL-4 are in line with the previously reported direct effect of Notch on IL-4 transcription (18, 22). A marked increase in Bcl6 protein levels was observed in T_{FH} cells of immunized control mice, and decreased Bcl6 protein levels were observed in the small number of detectable T_{FH} cells present in dLNs and PPs of $N1N2^{-/-}$ mice. Of note, decreased levels of Bcl6 were also observed in the $PD\text{-}1^{int}$ pre–T_{FH} cell population (Fig. 6C). GC T_{FH} cells have also been characterized by their high expression of GL7 (23). In contrast to control T_{FH} cells, which expressed high levels of PD-1 and BTLA and that could be subdivided into $GL7^-$ non-GC T_{FH} and $GL7^{high}$ GC T_{FH} cells, only very few $N1N2^{-/-}$ $PD\text{-}1^{high}$ $BTLA^{high}GL7^{high}$ or $GL7^-$ T_{FH} cells were detectable (Fig. 6D).

To further investigate the impact of Notch deficiency on the differentiation of T_{FH} cells, non–T_{FH}, pre–T_{FH}, and T_{FH} cells were similarly FACS sorted 7 d after the injection of *S. mansoni* eggs, which induces large number of T_{FH} cells, and expression of several markers associated with T_{FH} cells were analyzed by RT-PCR. A significant decrease in Bcl6 mRNA was observed in both $N1N2^{-/-}$ pre–T_{FH} and T_{FH} cells (Fig. 7A), in line with data obtained following NP-CGG immunization (Fig. 6B, 6C). High expression of IL-21 is another hallmark of T_{FH} cells. Accordingly, T_{FH} cells from control mice expressed high levels of IL-21 mRNA. In contrast, IL-21 mRNA expression was significantly impaired in $N1N2^{-/-}$ T_{FH} cells (Fig. 7B). The levels of c-Maf mRNA and other transcription factors present in T_{FH} cells were also reduced in the small population corresponding to $N1N2^{-/-}$ T_{FH} cells, whereas the small decrease in BATF mRNA observed in $N1N2^{-/-}$ T_{FH} cells was not statistically significant from that of control T_{FH} cells (Fig. 7C). Thus, the few T_{FH} cells present in dLNs of $N1N2^{-/-}$ mice do not express the characteristic array of markers defining differentiated T_{FH} cells.

Additionally, the phenotype of pre-T_{FH} cells was altered in $N1N2^{-/-}$ dLNs, with a markedly increased level of Blimp1 mRNA (Fig. 7D), as observed in NP-CGG–immunized mice (Fig. 6B). The frequency and number of $CD4^+PD\text{-}1^{int}$ pre–T_{FH} cells were similar between $N1N2^{-/-}$ and control mice, but the expression of CXCR5 was already decreased in that population for both NP-CGG– and *S. mansoni* eggs–immunized mice (Fig. 7E, 7F).

These results show that under physiological conditions, very few T_{FH} cells develop in $N1N2^{-/-}$ dLNs and that these T_{FH} cells lack most of the classical T_{FH} cell markers.

Discussion

In this study we have demonstrated that following immunization with T-dependent Ags or after exposure to Th2-inducing parasites, expression of Notch1 and Notch2 on T cells is essential for the differentiation of T_{FH} cells. Consequently, markedly reduced GC development as well as impaired maturation of high-affinity Ab-producing B cells was observed. This defect was shown to be T cell–intrinsic using mice with selective inactivation of Notch1 and Notch2 in their T cells, as well as mixed BM chimeras.

Increasing evidence implicates Notch signaling as a player in the differentiation of functional $CD4^+$ Th subsets. The impact of Notch signaling may affect Th cell differentiation and/or function (9–11). Using mice with a T cell–specific deletion of recombination signal-binding protein-J, Notch1, and Notch2, or with conditional expression of dominant-negative MAML1, an essential role for Notch in the development of a Th2 response was reported (22, 24, 25). Of note, in these studies, only IL-4 and its related isotype switching were measured as a readout for Th2 cell differentiation. Activation of IL-4 in T_{FH} cells was recently shown to be mainly dependent on a conserved IL-4 enhancer (CNS2) (26, 27), and Notch intracellular domain was previously shown to bind selectively to CNS2 in a reporter assay (17, 18, 28). Only very few T_{FH} cells developed in $N1N2^{-/-}$ mice following Th2-inducing stimuli, and we could not detect significant IL-4 levels in dLN T cells, suggesting that the absence of Notch signaling on T cells

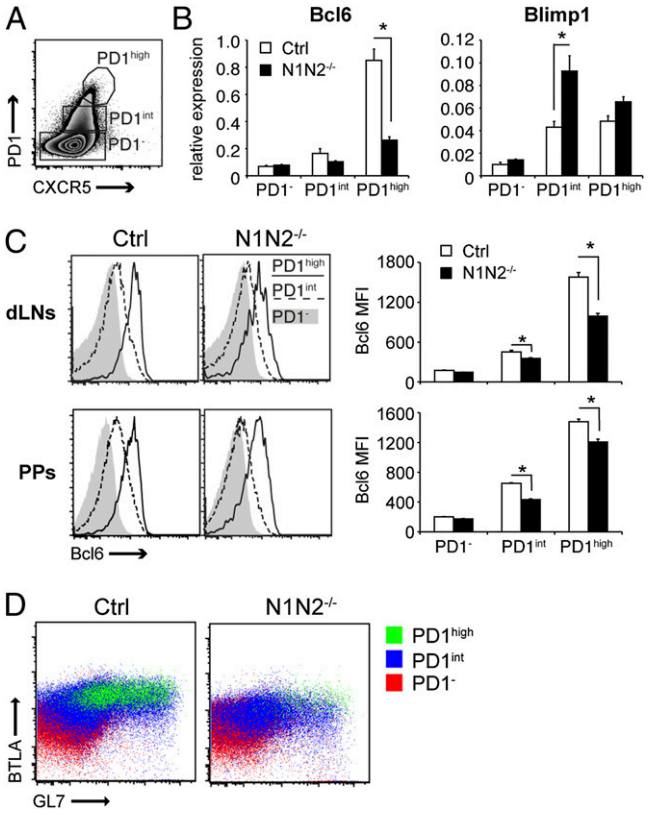

FIGURE 6. Notch expression on T cells affects the Bcl6/Blimp1 ratio. (**A**) PD-1^{high}CXCR5^{+}CD4^{+} T_{FH} cells, PD-1^{int}CXCR5^{-}CD4^{+} non-T_{FH} cells, and PD-1^{-}CXCR5^{-}CD4^{+} nonactivated cells were FACS sorted 7 d after NP-CGG immunization according to the gates. (**B**) Bcl6 and Blimp1 mRNA levels were analyzed in the three sorted populations by RT-PCR and normalized to HPRT mRNA expression. Results are represented as arbitrary units ± SEM for $n \geq 3$ mice/group. (**C**) Bcl6 expression was analyzed by intracellular staining in PPs and dLNs of naive and NP-CGG–immunized N1N2$^{-/-}$ and control mice, respectively. The mean fluorescence intensity (MFI) of Bcl6 expression is represented in histograms ± SEM for $n \geq 3$ mice/group. (**D**) GL7 expression was analyzed by flow cytometry in PD-1^{high}CXCR5^{+}CD4^{+} T_{FH} cells (green), PD-1^{int}CXCR5^{-}CD4^{+} non–T_{FH} cells (blue), and PD-1^{-}CXCR5^{-}CD4^{+} nonactivated cells (red) of *S. mansoni* eggs–immunized N1N2$^{-/-}$ and control mice. Representative FACS plots of $n = 5$ mice are given. All data are representative of at least two independent experiments. $^{*}p < 0.05$.

impairs IL-4 secretion by T_{FH} cells. In response to immunization or infection, Ag-restimulated N1N2$^{-/-}$ CD4^{+} T cells had impaired IL-4 secretion in line with the direct effect of Notch signaling on IL-4 transcription that also affects Th2 cells (18, 22), but they secreted high levels of the two Th2 cytokines IL-13 and IL-5, showing that unlike that of T_{FH} cells, the differentiation of Th2 cells can take place in the absence of Notch1 and Notch2 signaling. The differentiation of Th2 cells despite markedly reduced IL-4 secretion by CD4^{+} T cells suggests that the early IL-4 needed for Th2 differentiation originates from other cell types, as previously reported for *L. mexicana* infection (29).

In the presence of a functional GC, in mixed BM chimeras, a small number of Notch-deficient T cells developed into T_{FH} cells. Similarly, a small percentage of T_{FH} cells could also develop in the PPs of naive N1N2$^{-/-}$ mice that have a high number of GCs resulting from T-independent and T-dependent responses to gut microflora. During in vitro differentiation, the need for Notch signaling in Th cell differentiation can be bypassed in the presence of strong TCR signaling and high cytokine levels (12, 22, 30). Thus, a small proportion of T_{FH} cells may develop independently

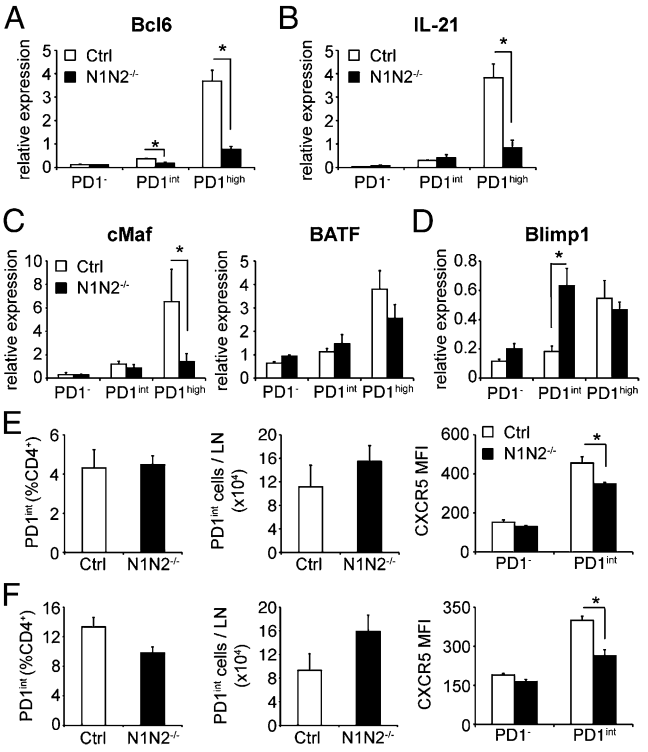

FIGURE 7. N1N2$^{-/-}$ T_{FH} cells do not express all the classical T_{FH} cell markers. (**A–D**) PD-1^{high}CXCR5^{+}CD4^{+} T_{FH} cells, PD-1^{int}CXCR5^{-}CD4^{+} non–T_{FH} cells, and PD-1^{-}CXCR5^{-}CD4^{+} nonactivated cells were FACS sorted 14 d after *S. mansoni* egg immunization according to the gates shown in Fig. 6A. Bcl6 (A), IL-21 (B), cMAF, BATF (C), and Blimp1 (D) mRNA levels were analyzed in the three sorted populations by RT-PCR. Results are normalized to HPRT mRNA expression and represented as arbitrary units ± SEM for $n \geq 3$ mice/group. (**E** and **F**) The mean frequency and number of PD-1^{int} cells is given for (E) NP-CGG– and (F) *S. mansoni* eggs–immunized mice, and PD-1^{-} and PD-1^{int} cells were analyzed for the expression of CXCR5, represented as the mean fluorescence intensity (MFI) ± SEM for $n \geq 3$ mice/group. $^{*}p < 0.05$.

of the need of Notch when sustained strong Ag signaling and/or chronic GCs are present.

We show that Notch signaling regulates the balance between Bcl6 and Blimp1 mRNA expression. Bcl6 is the master transcription factor of T_{FH} cells (20, 21, 31) and its expression is repressed by Blimp1, which is a transcription factor expressed by other Th effector cells. These two transcription factors regulate each other and determine commitment to either T_{FH} or other Th effector cells (20, 32). The impaired T_{FH} cell differentiation observed in the absence of Notch on T cells could be compatible with a positive action of Notch on Bcl6 and/or a negative one on Blimp1.

Repression of Blimp1 has been reported to allow Bcl6 expression, leading to T_{FH} cell differentiation (20). The lack of Notch signaling in parasite-exposed or NP-CGG–immunized mice modified the balance of Bcl6/Blimp1 mRNA in T cells, resulting in elevated levels of Blimp1 mRNA in pre-T_{FH} cells. A similar increase in Blimp1 mRNA was reported in Ag-specific CD4^{+} non-T_{FH} cells of Bcl6-deficient mice (33). Furthermore, the small expression of Bcl6 detected in pre–T_{FH} cells appeared already reduced in N1N2$^{-/-}$ pre–T_{FH} cells, correlating with reduced CXCR5 surface expression. These data suggest that Notch signaling is acting already at the pre–T_{FH} cell level.

In addition to Bcl6, the T_{FH} cell master transcription factor, other transcription factors have been involved in T_{FH} cell differentiation. Among these, BATF was reported to induce Bcl6 and c-Maf (14). The absence of Notch1 and Notch2 on T cells affected

both c-Maf and Bcl6 mRNA levels; however, only a slight reduction of BATF mRNA levels was observed, suggesting that the control of Notch signaling on T_{FH} cell differentiation does not act directly via the regulation of BATF expression. The exact Notch targets involved remain to be determined.

We further show that the few T_{FH} cells detectable in N1N2$^{-/-}$ mice lack the main classical markers of T_{FH} cells such as surface expression of CXCR5, high levels of PD-1, BTLA, and GL7 surface molecules, high levels of IL-21 mRNA and expression of Bcl6 and c-Maf transcription factors. Deficiency in IL-21 has been reported to have mild or no effect on T_{FH} cell differentiation, but IL-21 contributes to affinity maturation and GC formation (34–37). Absence of IL-21 results in a 50% reduction of GC B cell number whereas absence of T_{FH} cells results in total loss of GC (20, 21, 31). Thus, the absence of GC in immunized N1N2$^{-/-}$ mice results mostly from the impaired differentiation of T_{FH} cells, and the few N1N2$^{-/-}$ T_{FH} cells present are not secreting the IL-21 that could have induced some B cells to differentiate into GC B cells.

In summary, in this study we demonstrate an important contribution for Notch signaling in T_{FH} cell differentiation and consequently the development of GC B cells and generation of high-affinity Abs. These findings expand our understanding of the role of Notch in Th cell differentiation and function, and they have implications for understanding the underlying basis of Ab-mediated pathologies, thus providing insights as to how best they could be prevented or treated.

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Disclosures

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