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CD4$^+$ T follicular helper (T_{FH}) cells support germinal center (GC) reactions promoting humoral immunity. Dendritic cell (DC) diversification into genetically distinct subsets allows for specialization in promoting responses against several types of pathogens. Whether any classical DC (cDC) subset is required for humoral immunity is unknown, however. We tested several genetic models that selectively ablate distinct DC subsets in mice for their impact on splenic GC reactions. We identified a requirement for *Notch2*-dependent cDC2s, but not *Batf3*-dependent cDC1s or *Klf4*-dependent cDC2s, in promoting T_{FH} and GC B cell formation in response to sheep red blood cells and inactivated *Listeria monocytogenes*. This effect was mediated independent of *Il2ra* and several *Notch2*-dependent genes expressed in cDC2s, including *Stat4* and *Havcr2*. Notch2 signaling during cDC2 development also substantially reduced the efficiency of cDC2s for presentation of MHC class II-restricted antigens, limiting the strength of CD4 T cell activation. Together, these results demonstrate a nonredundant role for the *Notch2*-dependent cDC2 subset in supporting humoral immune responses.

dendritic cell | T follicular helper cell | germinal center

Classical dendritic cells (cDCs) (1) comprise two major lineages, distinguished by CD8α, CD24, XCR1, and CD103 expression by cDC1s and by CD4, Sirpα, and CD11b expression by cDC2s (2–4). cDC1s and cDC2s use distinct transcriptional programs for development and exert distinct functions in vivo (2, 3), exhibiting specialization for activation of CD8$^+$ and CD4$^+$ T cells, respectively (5–7). cDC1s require *Irf8*, *Batf3*, *Id2*, and *Nfil3* for development and cross-present antigens to CD8$^+$ T cells for antiviral and antitumor immunity (3). cDC2s develop independent of *Irf8*, *Batf3*, *Id2*, and *Nfil3*, but require *Irf4* for normal function (8). cDC2s require *Irf4* to support type II and type III immune responses (9–14). *Klf4* is required by cDC2s to sustain type II responses (15), while *Notch2* is required for type III responses in defense against *Citrobacter rodentium* (16, 17).

Recent work has suggested that cDC2s might mediate T follicular helper (T_{FH}) responses and germinal center (GC) reactions. T_{FH} cells were identified as follicular homing cells that express CXCR5 and PD-1 (18–20) and later found to require the transcription factor *Bcl6* for their differentiation (21–23). General depletion of cDCs using CD11c-DTR (24) or *Zbtb46*-DTR (25) impaired T_{FH} differentiation during *Toxoplasma gondii* infection (26), prevented humoral responses to allogeneic RBCs (27), and inhibited IgA class-switching in Peyer's patches (PPs) (28). Recently, secretion of the IL-2 receptor CD25 by cDCs was found to support T_{FH} differentiation in response to sheep red blood cell (SRBC) immunization (29). A separate study showed that CXCR5 expression by DCs and T cells was required for T_{FH} differentiation in response to the round worm *Heligmosomoides polygyrus* (30). Antigen targeting to cDC1s and cDC2s using anti-DEC205 and anti-DCIR2, respectively, showed that cDC2s are strong inducers of humoral responses (31). Furthermore, cDC2s were recently shown to be involved in some antibody responses, with $Itgax^{Cre}Irf4^{f/f}$ mice, but not $Batf3^{-/-}$ mice, showing impaired antibody responses to allogeneic RBCs (27). In addition, migration of cDC2s from the lung to the mediastinal lymph nodes was required to induce T_{FH} differentiation in response to soluble protein (32). Finally, $Batf3^{-/-}$ mice generate T_{FH} in response to immunization with SRBCs (29).

Here we extended our previous analysis of the in vivo function of *Notch2*-dependent cDCs by examining T_{FH} differentiation and GC responses in response to several forms of immunization. We find that the *Notch2*-dependent ESAM$^+$ cDC2 subset is required in two models of inducible T_{FH} differentiation and GC reaction in the spleen. Although the mechanism for this requirement remains unclear, we have extended the characterization of the *Notch2*-dependent transcriptional program in cDC2s, identifying several potential targets that merit evaluation, and identified an accompanying *Notch2*-dependent reduction of CD4$^+$ T cell response to antigens presented by DC2s acquired during terminal maturation.

Results

Notch2-Dependent DCs Are Required for GC Reactions Induced by SRBCs and Heat-Killed *Listeria*. We previously examined mice with conditional deletion of *Notch2* in the cDC2s induced by CD11c-Cre (24), finding that loss of ESAM$^+$ cDC2s is associated with reduced resistance to *C. rodentium* infection but normal responses to *T. gondii* and *Schistosoma mansoni* infection (15,

Significance

High-affinity antibody responses involve selection of B cells in the germinal center (GC) by cognate interactions with T follicular helper (T_{FH}) cells, which in turn must first be activated by classical dendritic cells (cDCs). We observe that *Notch2*-dependent cDC2s are required in vivo for induction of T_{FH} cells, GC B cells, and specific antibody production in response to sheep red blood cell (SRBC) immunization. Notch2 signaling impacted a broad transcriptional program in cDC2s both at homeostasis and after SRBC immunization, although we have not identified a target gene that mediates T_{FH} differentiation. Thus, Notch2 is a transcription factor that acts in cDCs and is selectively required for support of the GC reaction.

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Data deposition: Gene expression microarray data have been deposited in the Gene Expression Omnibus (accession no. GSE119242).

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17). cDC2s have been linked to priming $CD4^+$ T cells, but no studies have directly tested whether *Notch2*-dependent cDC2 function development of T_{FH} cells. We began by immunizing mice with SRBCs, which can induce responses by activating splenic $CD4^+$ cDC2s (33). Wild-type (WT) and CD11c-Cre^+ *Notch2*$^{f/f}$ mice (*Notch2*$^{\Delta 11c}$) were immunized i.p. and analyzed for T_{FH} cell differentiation and GC B cell formation (Fig. 1). As a control for specificity, we also tested *Batf3*$^{-/-}$ mice, which lack cDC1s (34). WT mice showed robust induction of PD-1^+ $CXCR5^+$ T_{FH} cell differentiation after immunization with SRBCs (Fig. 1 *A* and *B*). *Batf3*$^{-/-}$ mice also supported induction by SRBCs of T_{FH} cell development, even though they exhibit higher background before immunization compared with WT mice. In contrast, *Notch2*$^{\Delta 11c}$ mice showed no significant induction of T_{FH} cells in response to SRBC immunization (Fig. 1 *A* and *B*). Similarly, WT and *Batf3*$^{-/-}$ mice both showed induction of GL-7^+ Fas^+ GC B cells in response to SRBC immunization, while *Notch2*$^{\Delta 11c}$ mice showed no induction (Fig. 1 *C* and *D*). Furthermore, WT mice exhibited high titers of circulating anti-SRBC IgG1 titers at 17 d after immunization with SRBCs (Fig. 1*E*). Meanwhile, low anti-SRBC IgG1 titers were detected in sera in only one of five *Notch2*$^{\Delta 11c}$ mice immunized with SRBCs (Fig. 1*E*).

As another control, we examined mice with conditional deletion of *Klf4* in cDCs induced by CD11c-Cre (*Klf4*$^{\Delta 11c}$) (Fig. 1 *F* and *G*). We previously reported that *Klf4*$^{\Delta 11c}$ mice show a defect in protection against *S. mansoni* owing to decreased T_H2 responses (15). *Klf4*$^{\Delta 11c}$ mice were able to produce normal T_{FH} and GC B cell responses similar to littermate WT control mice in response to SRBC immunization (Fig. 1 *F* and *G*). These findings were confirmed by histological examination of spleen (Fig. 1*H*). Both WT and *Klf4*$^{\Delta 11c}$ mice generated robust $GL7^+$ GC reactions, but these were not evident in spleen sections of *Notch2*$^{\Delta 11c}$ mice.

We observed similar findings in spleens of WT and *Notch2*$^{\Delta 11c}$ mice immunized with heat-inactivated *Listeria monocytogenes* (ΔLM-OVA) (*SI Appendix*, Fig. S1). WT mice immunized with ΔLM-OVA generated robust T_{FH} cells and GC B cell reactions. In contrast, T_{FH} cells and GC B cell reactions were missing in immunized *Notch2*$^{\Delta 11c}$ mice. Similarly, histological analysis of splenic sections showed robust $GL7^+$ GC responses in WT mice, but not in *Notch2*$^{\Delta 11c}$ mice, immunized with ΔLM-OVA. Small intestine $CD103^+CD11b^+$ cDC2s, like splenic $ESAM^+$ cDC2s, also require Notch2 signaling for their development (17). Thus, we examined whether T_{FH} and GC reactions in PPs were impacted by the absence of this subset. However, we found similar percentages of T_{FH} and GC B cells in PPs from WT and *Notch2*$^{\Delta 11c}$ mice at steady state (*SI Appendix*, Fig. S2). Overall, these findings indicated a requirement for *Notch2*-dependent cDC2s in mediating GC responses in the spleen.

***Notch2*-Deficient cDC2s Have an Altered Transcriptional Response to SRBC Immunization.** Deletion of *Notch2* is known to inhibit the terminal maturation of splenic DCs (16, 17). However, whether the DC2s developing in *Notch2*$^{\Delta 11c}$ maintain their DC identity or acquire a macrophage phenotype has been unclear. We measured intracellular levels of Zbtb46 expression in WT and *Notch2*$^{\Delta 11c}$ DCs, since expression of this transcription factor distinguishes DCs from macrophages (35, 36), (*SI Appendix*, Fig. S3). We found that both DC1s and DC2s from *Notch2*$^{\Delta 11c}$ mice expressed Zbtb46 but not the macrophage surface marker F4/80 (37). Furthermore, the abundance of Zbtb46 was comparable in WT and *Notch2*$^{\Delta 11c}$ cDCs. These results imply that cDC2s in *Notch2*$^{\Delta 11c}$ mice retain their cDC identity.

We previously reported the transcriptional impact of Notch2 signaling during the terminal maturation of cDC2s at steady state, but not during DC activation (17). To examine the effect of Notch2 on gene expression during DC activation, we sorted

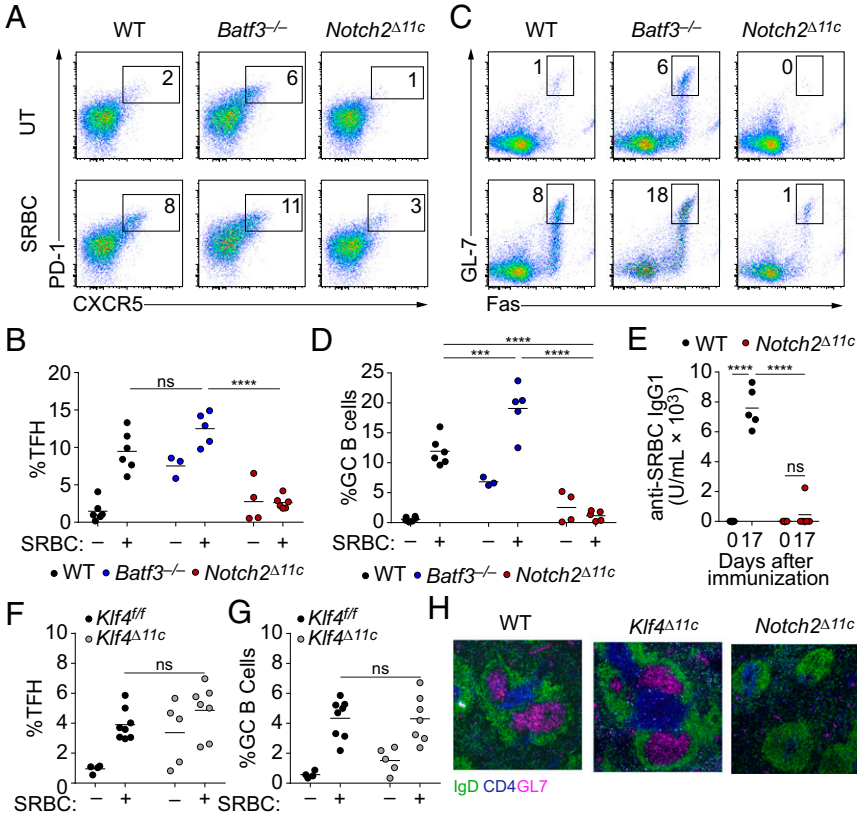

Fig. 1. Notch2-dependent DCs are required for GC reactions after immunization with SRBCs. (*A*) Representative flow cytometry analysis of T_{FH} cells ($B220^-TCR\text{-}\beta^+CD4^+CD8\text{-}CD44^+$) from the indicated mice 8 d after immunization with SRBCs. Shown as controls are untreated mice. (*B*) Quantification of the T_{FH} percentages in the indicated mice from *A*. (*C*) Representative flow cytometry analysis of GC B cells ($B220^+CD19^+IgD^-$) in spleens of the same mice as in *A*. (*D*) Quantification of GC B cells in the indicated mice from *C*. Each dot represents a biological replicate from three independent experiments. (*E*) Serum anti-SRBC IgG1 titers in WT and *Notch2* $^{\Delta 11c}$ mice at 17 d after immunization. Each dot represents a biological replicate. (*F* and *G*) Quantification of T_{FH} and GC B cells in *Klf4*$^{\Delta 11c}$ mice and littermate controls 8 d after immunization with SRBCs. Each dot represents a biological replicate from two independent experiments. (*H*) Microscopy of spleens from the indicated genotypes taken 8 d after immunization with SRBCs.

splenic cDC2s from untreated WT and *Notch2*$^{\Delta 11c}$ mice and from WT and *Notch2*$^{\Delta 11c}$ mice at 24 h after immunization with SRBCs and carried out global gene expression analysis (Fig. 2). We identified a fourfold induction of *Stat4* in WT cDC2s after treatment that did not occur in *Notch2*$^{\Delta 11c}$ cDC2s (Fig. 2*A*). At steady state, *Havcr2*, which encodes for Tim-3, a marker of T cell exhaustion (38), was fourfold higher in WT cDC2s compared with *Notch2*$^{\Delta 11c}$ cDC2s (Fig. 2*B*). In response to SRBC immunization, *Notch2*-deficient cDC2s, but not WT cDC2s, showed increased expression of CD14, the coreceptor for LPS, and *Ccl17*, a T-cell chemoattractant that acts through CCR4 (Fig. 2*C*). In addition, we observed an up-regulation of several members of the SLAM family of surface receptors in *Notch2*-deficient cDC2s compared with WT at steady state and on activation (Fig. 2*D*).

Tim-3 Expression by DC2 Requires Notch2 Signaling but Is Dispensable for GC Reactions. Tim-3 is a marker of differentiated T_H1 cells (39), and Tim-3 binding to galectin-9 promotes peripheral tolerance and inhibits T_H1 activity (40, 41). Tim-3 is also expressed on $CD8^+$ tumor-infiltrating lymphocytes (TILs), and Tim-3^+PD-1^+ TILs fail to proliferate or produce the proinflammatory cytokines IL-2, TNF, and IFN-γ (42). Combined anti-PD-1 and anti-Tim-3 immunotherapy promotes antitumor responses in mice bearing solid tumors (42). At steady state, cDC2s express Tim-3, and combined stimulation in vitro with LPS and galectin-9 induces TNF-α secretion by WT cDCs, but not Tim-3–deficient DCs (43). However, the regulation of Tim-3 expression on cDC2s, and whether it promotes GC reactions, have not been studied.

We first confirmed the reduction in *Havcr2* gene expression in *Notch2*$^{\Delta 11c}$ cDC2s at the level of protein expression. We previously found that loss of Notch2 signaling in cDCs impacted both splenic cDC subsets, including a reduction in ESAM expression by both cDC1s and cDC2s (17). Tim-3 expression was not reduced in *Notch2*$^{\Delta 11c}$ cDC1s (Fig. 3*A*) but was reduced sixfold in *Notch2*$^{\Delta 11c}$ cDC2s compared with WT controls (Fig. 3 *A* and *B*). In a murine model of hepatitis C virus, antibody-mediated blockade of Tim-3 enhanced T cell proliferation and IFN-γ production induced by HCV antigens (44). To test whether Tim-3 is required for the formation of GC reactions, we treated WT B6 mice with a blocking antibody against Tim-3 before SRBC immunization. We confirmed that Tim-3 blockade on cDC2s was effective by FACS analysis of DCs from mice treated with anti-Tim-3 or anti-trinitrophenol (clone 2A3) as isotype control in untreated controls and in mice immunized with SRBCs (*SI Appendix*, Fig. S4*A*). In mice treated with isotype antibody, we observed high expression of Tim-3 on DC2s irrespective of SRBC treatment. In contrast, the mean fluorescence intensity of Tim-3 staining on cDC2s was significantly reduced compared with that seen in mice treated previously with anti-Tim-3–blocking antibody (*SI Appendix*, Fig. S4*A*), showing that Tim-3 blockade was achieved. However, mice treated with anti-Tim-3 continued to support development of T_{FH} cells (Fig. 3 *C* and *D*) and GC B cells (Fig. 3 *C* and *D*) in response to SRBCs, similar to isotype-treated controls. These results suggest that, despite *Notch2*-dependent expression by DC2, Tim-3 is not required for T_{FH} or GC reactions.

Notch2-Dependent cDC2s Mediate GC Reactions Independently of CD25 or STAT4. Differentiation of naïve $CD4^+$ T cells into T_{FH} cells requires the inhibition of IL-2 signaling (45), which is promoted by cDC-specific secretion of CD25 (IL-2Rα). We hypothesized that the deficiency in GC reactions in *Notch2*$^{\Delta 11c}$ mice was due to impaired expression of CD25. First, we confirmed that cDC2s, but not cDC1s, induce CD25 after immunization with SRBC (Fig. 4*A*), as previously reported (29). However, we found that cDC2 expression of CD25 occurred independently of Notch2 signaling (Fig. 4*A*), suggesting the lack of GC reactions we observed in *Notch2*$^{\Delta 11c}$ mice was due to a distinct mechanism other than IL-2 inhibition.

In our gene expression analysis, we observed Notch2-dependent induction of STAT4 in cDC2s after SRBC immunization (Fig. 2*A*). STAT4 expression is reported to increase during DC maturation in vitro (46). In addition, monocyte-derived DCs generated by culture of peripheral blood monocytes with GM-CSF and IL-4 induce STAT4 after treatment with IFN-γ and LPS (47). However, the activity of STAT4 in DC function has not been studied in vivo. To test if STAT4 mediated the requirement for *Notch2* signaling in GC reactions, we immunized WT and *Stat4*$^{-/-}$ mice with SRBCs and analyzed induction of GC responses (Fig. 4*B*). However, we found no difference in the percentages of T_{FH} and GC B cells between WT and *Stat4*$^{-/-}$ mice (Fig. 4*B*). We confirmed these results by evaluating the formation of GCs in the

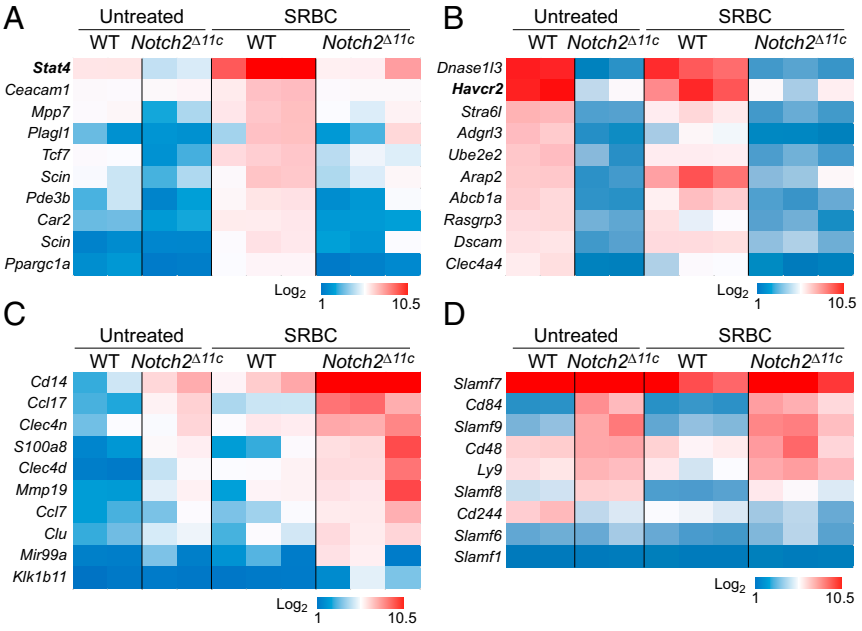

Fig. 2. Expression of Stat4, Tim-3, and SLAM family of proteins by DC2s is regulated by Notch2 signaling. Splenic DC2s (B220-CD11c$^+$MHC-II$^+$CD172a$^+$) were sorted from WT and *Notch2*$^{\Delta 11c}$ mice at 24 h after immunization with SRBCs or untreated controls and analyzed for gene expression by microarray. (*A* and *B*) Heatmap of expression of the top 10 probe sets down-regulated in *Notch2*$^{\Delta 11c}$ DC2s compared with WT after SRBC immunization (*A*) or at steady state (*B*). (*C*) Heatmap of expression of the top-10 transcripts up-regulated in *Notch2*$^{\Delta 11c}$ DC2s compared with WT DC2s after immunization with SRBCs. (*D*) Heatmap of SLAM family protein expression. Each column represents a biological replicate from two independent experiments.

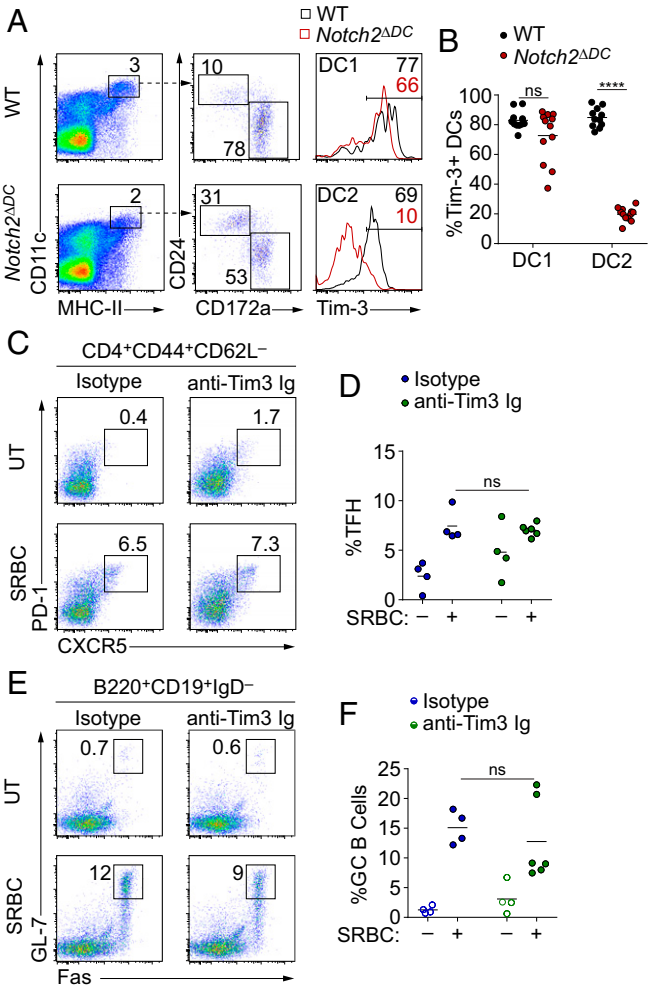

Fig. 3. Tim-3 expression on DC2s requires Notch2 signaling but is dispensable for GC reactions after immunization with SRBC. (*A*) Flow cytometry analysis of Tim-3 surface expression on DC1s ($CD24^+CD172a^-$) and DC2s ($CD24^-CD172a^+$) from WT or CD11c-Cre$^+$ *Notch2*$^{f/f}$ (*Notch2*$^{\Delta 11c}$) splenocytes. (*B*) Quantification of the percentage of Tim-3–expressing DCs from *A*. Each dot represents a biological replicate from five independent experiments. (*C*–*F*) WT mice treated with anti-Tim-3–blocking antibody (clone RMT3-23) or isotype IgG as a control were immunized with SRBCs and analyzed for GC reactions after 8 d. (*C*) Representative flow cytometry analysis of T_{FH} differentiation ($B220^-TCR\text{-}\beta^+CD4^+CD8^-CD44^+$). (*D*) Quantification of T_{FH} percentages in spleens of the indicated mice treated as in *E*. (*E*) Flow cytometry analysis of GC B cells ($B220^+CD19^+IgD^-$) in spleens of mice treated as in *E*. (*F*) Quantification of splenic GC B cells in mice treated as in *E*. Each dot represents a biological replicate.

spleen using histological analysis of $GL7^+$ GC B cells in splenic sections (Fig. 4*C*). Again, WT and *Stat4*$^{-/-}$ mice showed similar levels of GC formation in response to SRBC immunization.

***Notch2* Signaling Represses Antigen Presentation Efficiency of cDC2s.** Antigen presentation by cDCs is necessary to prime $CD4^+$ T cells against soluble antigens in vivo (48). Previous work has shown that cDCs are necessary and sufficient to initiate T_{FH} responses (26, 49). Antigen targeting to each cDC subset revealed that cDC2s are more efficient than cDC1 at presenting antigens on MHC-II molecules (6) and in promoting T_{FH} differentiation (31). To test whether *Notch2*$^{\Delta 11c}$ cDC2s have a defect in priming $CD4^+$ T cells, we examined their efficiency in presenting soluble antigens to OT-II cells. To do so, we cocultured sorted cDC2s from WT or *Notch2*$^{\Delta 11c}$ mice and CFSE-labeled OT-II T cells in the presence of varying concentrations of soluble OVA protein. We observed a 30-fold increase in the efficiency of antigen presentation by *Notch2*-deficient cDC2s compared with WT DC2s (Fig. 5*A*). We also tested whether Notch2 signaling repressed antigen presentation efficiency by measuring the endogenous $ESAM^+$ or $ESAM^-$ populations of cDC2s that are present in WT mice, since we previously determined that the $ESAM^+$ cDC2 subset represents the *Notch2*-dependent population in vivo (17). Therefore, we tested priming of OT-II T cells by endogenous $ESAM^+$ or $ESAM^-$ cDC2s (Fig. 5*B*). Notably, we found that the $ESAM^-$ cDC2 population was able to activate OT-II T cells at a significantly lower concentration of soluble OVA than the $ESAM^+$ cDC2 population. Furthermore, the efficiency of $ESAM^-$ cDC2s was similar to the efficiency of *Notch2*$^{\Delta 11c}$ cDC2s, both of which were more efficient than $ESAM^+$ cDC2s (Fig. 5*B*).

The SLAM family of receptors, which includes CD84 and Ly108, engage in high-affinity homotypic interactions (50). Signaling by these receptors is mediated by the SLAM-associated protein (SAP), encoded by *Sh2d1a*, which binds to the intracellular tail of SLAM receptors via an SH2 domain. T cells lacking SAP are unable to form stable interactions with their cognate B cell and *Sh2d1a*$^{-/-}$ mice lack T-dependent antibody responses due to deficient help from SAP-deficient T cells to B cells (51, 52). SAP-deficient OT-II cells cannot differentiate into T_{FH} cells and are unable to localize to the GC reaction (49). We found increased expression of several SLAM receptors by *Notch2*-deficient cDC2s (Fig. 2*D*). We wondered whether the increased efficiency in antigen presentation by *Notch2*-deficient cDC2s to OT-II cells (Fig. 5 *A* and *B*) was a result of increased SLAM expression and stabilization of DC:T interactions. Therefore, we examined the antigen presentation efficiency of sorted $ESAM^+$ and $ESAM^-$ cDC2s from spleens of WT and *Sh2d1a*$^{-/-}$ mice. We found that both WT and SAP-deficient $ESAM^-$ cDC2s induced OT-II proliferation with similar efficiency, and in both cases, there were significantly more efficient that their $ESAM^+$ DC2 counterparts (Fig. 5*C*). Thus, while *Notch2*-deficient DC2s are more efficient at priming $CD4^+$ T cells to soluble antigens, this activity was independent of SLAM signaling.

Discussion

We previously reported a requirement for *Notch2*-dependent cDC2s in innate defense against *C. rodentium* mediated by IL-23 (17). A role for *Notch2*-dependent cDC2s in regulating adaptive immunity was implied by a twofold reduction in T_H17 cells in intestinal lamina propria in *Notch2*$^{\Delta 11c}$ mice at steady state (16). However, *Notch2*-dependent cDC2s are not required for T_H17 responses to *Streptococcus pyogenes* (53), to segmented filamentous bacteria (SFB) (54), T_H2 responses against *S. mansoni* (15), or development of peripheral T_{reg} cells (55). This study identifies a role *Notch2*-dependent DC2s in mediating T_{FH} differentiation and splenic GC responses to certain modes of immunization.

T_{FH} differentiation occurs rapidly after immunization (56). T_{FH} cells are distinguished by expression of CXCR5 and down-regulation of CD25 (57). T cell priming by DCs proceeds through three phases, beginning with short T:DC interactions, followed by long-term T:DC conjugates that induce cytokine production and T cell proliferation, and finally a return to short-term contact and T cell migration (58). The long-term T:DC interactions leading up to the third stage are required for T_{FH} differentiation (59). In some settings, DCs are sufficient for T_{FH} differentiation. With high antigen concentrations, B cells appear dispensable for T_{FH} differentiation, and persistent antigen presentation by DCs appears to be sufficient to induce full T_{FH} maturation (26, 49). Higher antigen levels and longer dwelling times decrease T_H1 output and promote T_{FH} numbers (60),

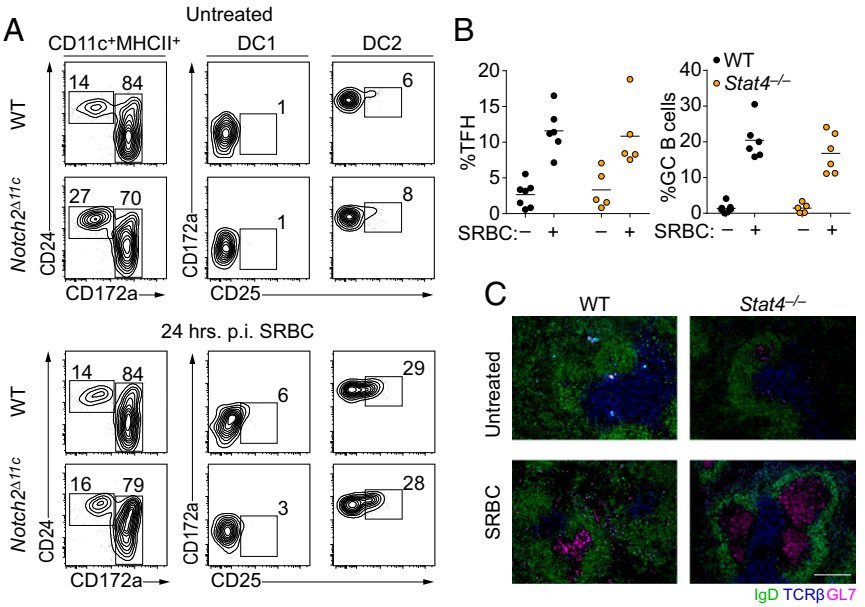

Fig. 4. Notch2-dependent DCs do not require CD25 or Stat4 to induce GC reactions. (*A*). Representative flow cytometry analysis of CD25 expression on splenic cDCs from WT or *Notch2*$^{\Delta 11c}$ mice at 24 h after immunization with SRBCs. Shown are two-color histograms for CD25 expression on DCs from untreated mice as controls. (*B*) WT and *Stat4*$^{-/-}$ mice were immunized with SRBCs and analyzed for GC reactions after 8 d. Quantification of T_{FH} and GC B cells. Each dot represents a biological replicate from three independent experiments; unimmunized mice are shown as controls. (*C*) Microscopy of splenic GC reactions in WT and *Stat4*$^{-/-}$ mice immunized with SRBCs 8 d earlier.

perhaps suggesting that stronger TCR signaling favors T_H1 over T_{FH} differentiation.

Notch2 signaling during cDC2 development appears to decrease the efficiency of antigen presentation, an effect evident in direct comparisons between endogenous ESAM$^+$ and ESAM$^-$ cDC2 populations in the spleen. Splenic ESAM$^+$ cDC2s are Notch2-dependent and develop through encounters with Delta-like 1 (61). Conceivably, the greater efficiency of antigen presentation by *Notch2*-deficient DC2s provides stronger TCR signaling, which might not be favorable to T_{FH} differentiation. Loss of Notch2 on cDC2s increased the expression of multiple SLAM proteins, which stabilize B/T conjugates that promote T_{FH} maturation in GCs (49). However, the increased OT-II proliferation induced by *Notch2*$^{\Delta 11c}$ DC2s was not dependent on SAP, the SLAM signaling mediator (51, 52), suggesting that changes in other molecules may be involved.

We previously reported an approximate twofold reduction in the total number of cDC2s in spleens from Notch2Δ11c mice compared with littermate controls (17). Conceivably, this reduction in cDC2s alone might be the cause of impaired GC reactions in response to SRBCs. However, WT and *Notch2*-deficient cDC2s showed qualitative changes in gene expression. Notch2 signaling has a pleiotropic impact in the development and function of cDC2s, which might contribute to the absence of GC reactions reported here. We previously reported that at steady state, Notch2-deficient cDC2s are reduced threefold in splenic and gut tissues, and in a competitive setting, these cDC2s are outcompeted by their WT counterparts (17). However, we also find that after activation with SRBCs, more than 2,000 genes are differentially expressed between WT and *Notch2*$^{\Delta 11c}$ cDC2s. While we have ruled out the involvement of some Notch2 targets, like STAT4 and Tim-3, in the induction of GC reactions in response to SRBCs, other pathways regulating cDC2 activation of T_{FH} and GC B cells may be dependent on Notch2 activity. For example, GC reactions require the correct localization of DCs, B cells, and CD4$^+$ T cells. The G protein-coupled receptor Ebi2 (*GPR183*) is involved in colocalization of activated cDC2s, T cells, and B cells at the follicle–T zone interface in the spleen (62–64). Correct positioning of cDC2s supports the induction of T_{FH} and GC reactions (65). cDC2 localization within the MZ bridging channels also requires Notch2 signaling (17). While *Notch2*-deficient cDC2s can prime CD4$^+$ T cells in vitro, the lack of ESAM$^+$ cDC2s located in the bridging channel may impair T_{FH} differentiation from lack of proximity to the B cell follicle in vivo. Further work is needed to determine the involvement of Notch2 signaling in DC localization and its role in T_{FH} development.

Materials and Methods

Mice. The following mice were acquired from Jackson Laboratories: CD11c-Cre [B6.Cg-Tg(Itgax-cre)1–1Reiz/J], *Notch2*^{fff} (B6.129S-Notch2tm3Grid/J), CD45.1$^+$ B6.SJL (B6.SJL-PtprcaPepcb/BoyJ), *Stat4*$^{-/-}$ (C57BL/6J-Stat4em3Adiuj/J), and *Sh2d1a*$^{-/-}$ (B6.129S6-Sh2d1atm1Pls/J). *Notch2*^{fff} mice were crossed to CD11c-Cre mice to generate Notch2Δ11c mice. The generation of *Zbtb46*$^{gfp/gfp}$ and *Batf3*$^{-/-}$ mice has been described previously (34, 36). *Klf4*^{fff} mice were obtained from the Mutant Mouse Resource and Research Center (MMRRC; line 29877) and crossed to CD11c-Cre mice to generate *Klf4*$^{\Delta 11c}$ mice. All mice were maintained on the C57BL/6 background in a specific pathogen-free animal facility following institutional guidelines and with protocols approved by the Animal Studies Committee at Washington University in St. Louis. Experiments were performed with mice age 8–12 wk using sex-matched littermates.

Antibodies and Flow Cytometry. Cells were kept at 4 °C while being stained in PBS with 0.5% BSA and 2 mM EDTA in the presence of CD16/32 Fc block (BD; clone 2.4G2). For intracellular flow cytometry, cells were stained for surface markers, permeabilized, and fixed with the transcription buffer set (BD) following the manufacturer's instructions. Cells were then stained for intracellular Zbtb46 expression at 4 °C on ice. Cells were analyzed on a FACS Canto II and sorted on a FACS Aria Fusion flow cytometers (BD). Data were

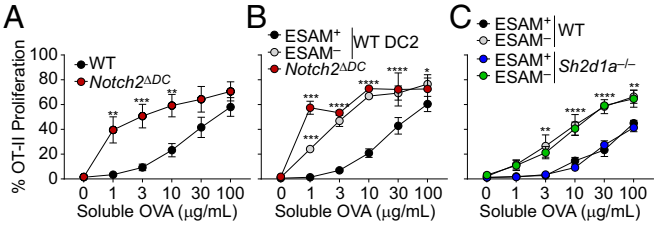

Fig. 5. Notch2 signaling programs DC2 for reduced priming efficiency independent of SLAM signaling. (*A*) Soluble antigen presentation assay of WT and *Notch2*$^{\Delta 11c}$ sorted DC2s. Shown is the mean percentage of CFSE dilution in OT-II cells at 3 d after coculture. (*B*) Splenic ESAM$^+$ and ESAM$^-$ DC2s from WT mice were cocultured with OT-II CD4 T cells as in *A*. The mean percentage CFSE dilution in OT-II cells after 3 d of culture is shown. $n = 6$ biological replicates for ESAM$^+$ and ESAM$^-$ WT DC2s; $n = 2$ for Notch2Δ11c DC2s. (*C*) Splenic ESAM$^+$ and ESAM$^-$ DC2s from WT and *Sh2d1a*$^{-/-}$ mice were cocultured with OT-II T cells as in *A*.

analyzed with FlowJo software (Tree Star). Antibodies and other staining materials are described in *SI Appendix, Materials and Methods*.

Statistical Analysis. In the figures, error bars indicate the SEM. Statistical analyses were performed using two-way ANOVA with Sidak's multiple-comparison test. All statistical analyses were performed using Prism (GraphPad Software).

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