Notch2-dependent DC2s mediate splenic germinal center responses

Carlos G. Briseño,
Ansuman T. Satpathy,
Jesse T. Davidson IV,
Stephen T. Ferris,
Vivek Durai,
Prachi Bagadia,
Kevin W. O’Connor,
Derek J. Theisen,
Theresa L. Murphy,
and Kenneth M. Murphy

CD4+ T follicular helper (TFH) 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 TFH and GC B cell formation in response to sheep red blood cells and inactivated Listeria monocytogenes. This effect was mediated independent of IIf4a 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.

Significance

High-affinity antibody responses involve selection of B cells in the germinal center (GC) by cognate interactions with T follicular helper (TFH) 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 TFH 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, identifying several potential targets that merit evaluation, and identified an accompanying Notch2-dependent reduction of CD4+ T cell response to antigens presented by DCs 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+ cDC2 subset is required with reduced resistance to C. rodentium infection but normal responses to T. gondii and Schistosoma mansoni infection (15, 29). Recent work has suggested that cDC2s might mediate T follicular helper (TFH) responses and germinal center (GC) reactions. TFH 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 Zbtb6-B6-DTR (25) impaired TFH 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 TFH 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 TFH 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 cDCs are strong inducers of humoral responses (31). Furthermore, cDC2s were recently shown to be involved in some antibody responses, with ItgaxCreIIf4a/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 TFH differentiation in response to soluble protein (32). Finally, Batf3+/− mice generate TFH in response to immunization with SRBCs (29).

Here we extended our previous analysis of the in vivo function of Notch2-dependent cDCs by examining TFH 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 TFH 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 DCs acquired during terminal maturation.
cDC2s have been linked to priming CD4+ T cells, but no studies have directly tested whether Notch2-dependent cDC2 function development of TFH cells. We began by immobilizing mice with SRBCs, which can induce responses by activating splenic CD4+ cDC2s (35). Wild-type (WT) and CD11c-Cre+ Notch2Δ11c mice (Notch2Δ11c) were immunized i.p. and analyzed for TFH cell differentiation and GC B cell formation (Fig. 1). As a control for specificity, we also tested Batf3Δ1c mice, which lack cDC1s (34). WT mice showed robust induction of PD-1+ CXCR5+ TFH cell differentiation after immunization with SRBCs (Fig. 1A and B). Batf3Δ1c mice also supported induction by SRBCs of TFH cell development, even though they exhibit higher background before immunization compared with WT mice. In contrast, Notch2Δ11c mice showed no induction of TFH cells in response to SRBC immunization (Fig. 1A and B). Similarly, WT and Batf3Δ1c mice both showed induction of GL-7+ Fas+ GC B cells in response to SRBC immunization, while Notch2Δ11c mice showed no induction (Fig. 1C and D). Furthermore, WT mice exhibited high titers of circulating anti-SRBC IgG1 titers at 17 d after immunization with SRBCs (Fig. 1E). Meanwhile, low anti-SRBC IgG1 titers were detected in sera in only one of five Notch2Δ11c mice immunized with SRBCs (Fig. 1E).

As another control, we examined mice with conditional deletion of Klf4 in cDCs induced by CD11c-Cre (Klf4Δ11c) (Fig. 1F and G). We previously reported that Klf4Δ11c mice show a defect in protection against S. mansoni owing to decreased TFH responses (15). Klf4Δ11c mice were able to produce normal TFH and GC B cell responses similar to littermate control mice in response to SRBC immunization (Fig. 1F and G). These findings were confirmed by histological examination of spleen (Fig. 1H). Both WT and Klf4Δ11c mice generated robust GL-7+ GC reactions, but these were not evident in spleen sections of Notch2Δ11c mice.

We observed similar findings in spleens of WT and Notch2Δ11c mice immunized with heat-inactivated Listeria monocytogenes (ΔLM-OVA) (SI Appendix, Fig. S1). WT mice immunized with ΔLM-OVA generated robust TFH cells and GC B cell reactions. In contrast, TFH cells and GC B cell reactions were missing in immunized Notch2Δ11c mice. Similarly, histological analysis of splenic sections showed robust GL-7+ GC responses in WT mice, but not in Notch2Δ11c 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 TFH and GC reactions in PPs were impacted by the absence of this subset. However, we found similar percentages of TFH and GC B cells in PPs from WT and Notch2Δ11c 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 cDCs 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 Notch2Δ11c maintain their DC identity or acquire a macrophage phenotype has been unclear. We measured intracellular levels of Zbtb46 expression in WT and Notch2Δ11c 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 Notch2Δ11c mice expressed Zbtb46 but not the macrophage surface marker F4/80 (37). Furthermore, the abundance of Zbtb46 was comparable in WT and Notch2Δ11c cDC2s. These results imply that cDC2s in Notch2Δ11c mice retain their DC 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 TFH cells (B220+CD4+CD3−CD44−CD8−CD19−IgD+CD45R−R71.1−R71.1+) from the indicated mice 8 d after immunization with SRBCs. Shown as controls are untreated mice. (B) Quantification of the TFH percentages in the indicated mice from A. (C) Representative flow cytometry analysis of GC B cells (B220+CD19−IgD−IgM−) 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 Notch2Δ11c mice at 17 d after immunization. Each dot represents a biological replicate from two independent experiments. (F) Microscopy of spleens from the indicated genotypes taken 8 d after immunization with SRBCs.

Briseño et al. PNAS | October 16, 2018 | vol. 115 | no. 42 | 10727
splenic cDCs from untreated WT and Notch2Δ11c mice and from WT and Notch2Δ11c 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 cDCs after treatment that did not occur in Notch2Δ11c cDCs (Fig. 2A). At steady state, Haver2, which encodes for Tim-3, a marker of T cell exhaustion (38), was fourfold higher in WT cDCs compared with Notch2Δ11c cDCs (Fig. 2B). In response to SRBC immunization, Notch2-deficient cDCs, but not WT cDCs, showed increased expression of CD14, the coreceptor for LPS, and Ccl17, a T-cell chemotactar that acts through CCR4 (Fig. 2C). In addition, we observed an up-regulation of several members of the SLAM family of surface receptors in Notch2-deficient cDCs compared with WT at steady state and on activation (Fig. 2D).

**Tim-3 Expression by DC2 Requires Notch2 Signaling but is Dispensable for GC Reactions.** Tim-3 is a marker of differentiated T\(_h\) cells (39), and Tim-3 binding to galectin-9 promotes peripheral tolerance and inhibits T\(_h\)1 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, cDCs 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 cDCs, and whether it promotes GC reactions, have not been studied.

We first confirmed the reduction in Haver2 gene expression in Notch2Δ11c cDCs 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 Notch2Δ11c cDCs (Fig. 3A) but was reduced sixfold in Notch2Δ11c cDCs compared with WT controls (Fig. 3 A and B). In a murine model of hepatitis C virus, antibody-mediated depletion 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 cDCs 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). In mice treated with isotype antibody, we observed high expression of Tim-3 on cDCs irrespective of SRBC treatment. In contrast, the mean fluorescence intensity of Tim-3 staining on cDCs was significantly reduced compared with that seen in mice treated previously with anti-Tim-3-blocking antibody (SI Appendix, Fig. S4), showing that Tim-3 blockade was achieved. However, mice treated with anti-Tim-3 continued to support development of T\(_h\)1 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\(_h\)1 or GC reactions.

**Notch2-Dependent cDC2 Mediate GC Reactions Independently of CD25 or STAT4.** Differentiation of naïve CD4\(^+\) T cells into T\(_h\)1 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 Notch2Δ11c mice was due to impaired expression of CD25. First, we confirmed that cDCs, but not cDC1s, induce CD25 after immunization with SRBC (Fig. 4A), as previously reported (29). However, we found that cDC2 expression of CD25 occurred independently of Notch2 signaling (Fig. 4A), suggesting the lack of GC reactions we observed in Notch2Δ11c 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 cDCs after SRBC immunization (Fig. 4). 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 Stat4ΔΔ mice with SRBCs and analyzed induction of GC reactions (Fig. 4B). However, we found no difference in the percentages of T\(_h\)1 and GC B cells between WT and Stat4ΔΔ mice (Fig. 4B). We confirmed these results by evaluating the formation of GCs in the
GC B cells in splenic mice showed similar cDC2 population was able to mice lack T-dependent antibody responses due or ESAM populations of cDC2s that are and T cells to soluble antigens, this activity was in- responses Notch2 DC2 counterparts mice and CFSE-labeled OT-II T cells in cells and T cells, we examined their efficiency in presenting soluble cDC2s induced OT-II pro- B differentiation occurs rapidly after immunization (56). mice. We found that differentiation, and persistent antigen pre- (31). To test whether cDC2s (Fig. 5 a) Quantification of splenic GC B cells in mice treated as in (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^eff^ (Notch2^{Δ11c}) 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 Tim-3 differentiation (B220^+^CD4^−^CD8^+^DC4^+^CD44^−^). (D) Quantification of Tim-3 percent- ages 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.

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^{Δ11c} 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^{Δ11c} 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. 5A). 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 popu- lation in vivo (17). Therefore, we tested priming of OT-II T cells by endogenous ESAM^+^ or ESAM^−^ cDC2s (Fig. 5B). 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^{Δ11c} cDC2s, both of which were more efficient than ESAM^−^ cDC2s (Fig. 5B).

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 intracel- lular 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 in- creased expression of several SLAM receptors by Notch2-deficient cDC2s (Fig. 2D). We wondered whether the increased efficiency in antigen presentation by Notch2-deficient cDC2s to OT-II cells (Fig. 5A and B) was a result of increased SLAM expression and stabilization of DCT 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. 5C). Thus, while Notch2-deficient DC2s are more efficient at priming CD4^+^ T cells to soluble antigens, this activity was inde- pendent 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_{FH}17 cells in intestinal lamina propria in Notch2^{Δ11c} mice at steady state (16). However, Notch2-dependent cDC2s are not required for T_{FH}17 responses to Streptococcus pyogenes (53), to segmented filamentous bacteria (SFB) (54), T_{FH}2 responses against S. mansonii (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_{FH}1 output and promote T_{FH} numbers (60),
perhaps suggesting that stronger TCR signaling favors T<sub>FH</sub> over T<sub>FH</sub> differentiation.

Notch2 signaling during cDC2 development appears to decrease the efficiency of antigen presentation, an effect evident in direct comparisons between endogenous ESAM<sup>+</sup> and ESAM<sup>-</sup> cDC2 populations in the spleen. Splenic ESAM<sup>+</sup> cDC2s are Notch2-dependent and develop through encounters with Delta-like 1 (61). Conceivably, the greater efficiency of antigen presentation by Notch2-deficient DCs provides stronger TCR signaling, which might not be favorable to T<sub>FH</sub> differentiation. Loss of Notch2 on cDC2s increased the expression of multiple SLAM proteins, which stabilize B/T conjugates that promote T<sub>FH</sub> maturation in GCs (49). However, the increased OT-II proliferation induced by Notch2<sup>−/−</sup> cDC2s 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 Notch2<sup>Δ11c</sup> 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<sup>Δ11c</sup> 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<sub>FH</sub> and GC B cells may be dependent on Notch2 activity. For example, GC reactions require the correct localization of DCs, B cells, and CD4<sup>+</sup> 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<sub>FH</sub> and GC reactions (65). cDC2 localization within the MZ bridging channels also requires Notch2 signaling (17). While Notch2-deficient cDC2s can prime CD4<sup>+</sup> T cells in vitro, the lack of ESAM<sup>+</sup> cDC2s located in the bridging channel may impair T<sub>FH</sub> 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<sub>FH</sub> development.

**Materials and Methods**

**Mice.** The following mice were acquired from Jackson Laboratories: CD11c-Cre [B6.Cg-Tg(Itgax-cre)1–1Reiz/J], Notch2<sup>Δ11c</sup> (B6.129-Notch2tm3Girdj/J), CD45.1<sup>+</sup> B6.5J (B6.SL-Ptpn10<sup>–/–</sup> mice, C57BL/6−<sup>−</sup>Stat4em3Adiuj/J), and Sh2d1a<sup>ΔDC</sup> (B6.129S6-Sh2d1atm1Pls/J). Mice were crossed to CD11c-Cre mice to generate Notch2<sup>Δ11c</sup> mice. The generation of ESAM<sup>−/−</sup> mice has been described previously (34, 36).

**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). Antibodies and their specific markers were used for intracellular staining as indicated in the Materials and Methods section. Cells were analyzed using a FACSCanto II and sorted on a FACSAria Fusion flow cytometers (BD). Data were analyzed using FlowJo software.

**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<sup>Δ11c</sup> 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<sup>−/−</sup> mice were immunized with SRBCs and analyzed for GC reactions after 8 d. Quantification of T<sub>FH</sub> 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<sup>−/−</sup> mice immunized with SRBCs 8 d earlier.
Materials and Methods

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).

Acknowledgments

We thank the Genome Technology Access Center, Department of Genetics, Washington University School of Medicine in St. Louis for help with genomic analysis. The Center is supported by Cancer Center Support Grant P30 CA091842 from the National Cancer Institute and by Institute of Clinical and Translational Sciences/Clinical and Translational Science Award UL1 TR000448 from the National Center for Research Resources. A.T.S. is supported by a Parker Bridge Scholar Award from the Parker Institute for Cancer Immunotherapy. V.D. and D.J.T. are supported by the National Institutes of Health (Grants F30DK108494 and T32 AI007163-40, respectively). J.T.D. is supported by the National Cancer Institute (Grant T32 CA 09621), and P.B. is supported by the National Science Foundation (Grant DGE-1143954). K.M.M. is supported by the Howard Hughes Medical Institute.