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*JCI Insight*. 2020. <https://doi.org/10.1172/jci.insight.134508>.

Research In-Press Preview Immunology

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**DOCK8 is essential for LFA-1 dependent positioning of T follicular helper cells in germinal centers**

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**Key words:** DOCK8, T follicular helper cells, germinal center, B cells, LFA-1

## **ABSTRACT**

T follicular helper (Tfh) cell migration into germinal centers (GC) is essential for the generation of GC B cells and antibody responses to T dependent (TD) antigens. This process requires interactions between LFA-1 on Tfh cells and ICAMs on B cells. The mechanisms underlying defective antibody responses to TD antigens in DOCK8 deficiency are incompletely understood. We show that mice selectively lacking DOCK8 in T cells have impaired IgG antibody responses to TD antigens, decreased GC size, and reduced numbers of GC B cells. However, they develop normal numbers of Tfh cells with intact capacity for driving B cell differentiation into a GC phenotype *in vitro*. Notably, migration of DOCK8 deficient T cells into GCs is defective. Following TCR/CD3 ligation, DOCK8 deficient T cells have impaired LFA-1 activation and reduced binding to ICAM-1. DOCK8 is important for LFA1-dependent positioning of Tfh cells in GCs, and thereby the generation of GC B cells and IgG antibody responses to TD antigen.

## INTRODUCTION

The antibody response to T cell dependent (TD) antigens requires interactions between T follicular helper (Tfh) cells and B cells. Tfh cell differentiation is initiated upon T cell recognition of antigen presented by dendritic cells (DCs) in the interfollicular areas of lymph nodes (LNs) (1). Activated T cells migrate towards the B cell follicle, where they interact with B cells. This leads to the expression of Tfh specific markers (2), including ICOS and PD1 (3-5). The interaction of ICOS with ICOS-L on B cells induces expression of the transcription factor BCL6 (4, 5). In turn, BCL6 upregulates CXCR5 expression on Tfh cells (3, 4). CXCR5 drives the migration of Tfh cells towards CXCL13 produced by stromal cells in B cell follicles (6). Adhesion molecules, including LFA-1, are important for T cell migration and retention in LNs (7, 8). Furthermore, the interaction between LFA-1 and ICAM-1/2 on B cells is a critical for productive Tfh-B cell interactions leading to B cell clonal expansion (9).

In the GC, Tfh and antigen specific B cells that have acquired antigen from follicular dendritic cells form strong, but short-lived contacts (10, 11). B cell signals are needed for the maintenance of the Tfh phenotype (12) and upregulate Tfh cell expression of IL-4 and IL-21 (11). Tfh cell production of IL-4 and IL-21, as well as the interaction between CD40L on Tfh cells and CD40 on B cells, are important for GC B cell proliferation, differentiation, BCL6 expression, and survival (13-15). GC B cells expressing a high affinity B cell receptor for antigen compete more effectively for survival signals delivered by Tfh cells (16).

DOCK8 deficiency is a primary immune deficiency characterized by recurrent infections and diminished responses to vaccines (17-19). Similarly, the antibody response to TD antigens is impaired in *Dock8*<sup>-/-</sup> mice. While there is data to suggest that B cells contribute to the impaired antibody response to TD antigens in DOCK8 deficiency (20),

there is less known about the role of T cells. We show that mice with selective DOCK8 deficiency in T cells mount poor IgG antibody responses to TD antigens, have impaired GC formation, and reduced numbers of GC B cells despite normal numbers of Tfh cells that are able to normally drive B cell differentiation *in vitro*. We demonstrate that activated DOCK8 deficient T cells have impaired LFA-1 activation and defective migration into GCs

## RESULTS

**DOCK8 expression in T cells is essential for a normal IgG antibody response to TD antigens.** *Dock8*<sup>-/-</sup> and *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice, that lack DOCK8 only in T cells (Supplemental Figure 1A), and their controls were immunized in the hocks with the TD antigen TNP-KLH. Serum anti-TNP IgG, but not IgM, antibody titers were significantly decreased in *Dock8*<sup>-/-</sup> and *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice (Figure 1A and Supplemental Figure 1B). Serum anti-TNP IgE antibody titers and IgE levels were not significantly different between *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice and their controls (Supplemental Figure 1C). This may be explained by known differences in the differentiation of B cells into IgE versus IgG-secreting plasma cells (21). CD4<sup>+</sup> T cells from TNP-KLH immunized *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice proliferated and secreted IL-2 and IFN- $\gamma$  normally in response to *in vitro* stimulation with KLH, demonstrating that they do not have a global activation defect (Supplemental Figure 1D, E).

The decreased IgG antibody response of *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice to TNP-KLH was specific neither for antigen nor route of immunization. *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice had a decreased anti-NP IgG antibody response to hock immunization with NP-OVA in alum (Figure 1B), as well as decreased TNP- and OVA-specific IgG antibody responses to intraperitoneal immunization with TNP-KLH and OVA in alum (Figure 1B). These findings demonstrate that DOCK8 expression in T cells is important for the IgG antibody response to TD antigen.

**DOCK8 expression in T cells is essential for normal GC formation and generation of GC B cells.** GC development in draining LNs is first observed 2-3 days following immunization with TD antigen (12), and a mature GC forms by approximately

seven days (22). GC formation in the popliteal LN of *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice was assessed by immunofluorescence microscopy on day 10 post-immunization in the hock with TNP-KLH in alum. GCs were significantly reduced in size in the LNs of *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice compared to *Cd4-Cre<sup>Tg</sup>* controls (Figure 2A). GCs are important for the formation of high affinity IgG antibody (23). High affinity antibodies against TNP were decreased in *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice compared to *Cd4-Cre<sup>Tg</sup>* controls 14 days after immunization with TNP-KLH (Figure 2B), suggesting that the smaller GCs formed in *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice are less efficient at promoting antibody affinity maturation.

There was a significant decrease in the percentage and number of mature GC B cells in the draining LNs of *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice (Figure 2C). Surface expression of GL7 and FAS on GC B cells was significantly reduced in *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice (Figure 2D). Surface levels of GL7 and FAS on GC B cells correlate with their maturation state (24), their reduction on GC B cells from *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice may due to a block in GC B cell maturation or could reflect an artificial lowering of the MFIs due to contamination of the few “true” GC B cells with non-GC B cells. GC B cells are highly proliferating cells and express BCL6 (25, 26). Intracellular FACS staining revealed that the percentage of proliferating BCL6<sup>+</sup>Ki-67<sup>+</sup> B cells in draining LNs was severely reduced in *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice (Figure 2E). However, intracellular expression of BCL6 and Ki-67 in BCL6<sup>+</sup> B cells was not affected (Figure 2F). These data indicate that DOCK8 expression in T cells is critical for supporting the differentiation and/or maintenance of GC B cells.

The differentiation of GC B cells following immunization with TD antigen is preceded by the generation of FAS<sup>int</sup>GL7<sup>lo</sup>IgD<sup>hi</sup>CCR6<sup>hi</sup> pre-GC B cells in the interfollicular regions of the LN driven by the interaction between antigen specific T and B cells. Pre-GC B cells

subsequently enter the follicles to form GC B cells that upregulate FAS and GL7 expression and downregulate CCR6 and IgD expression. The percentage of pre-GC B cells in draining LNs peaks on day 2 post-immunization (24, 27-29). The percentage of pre-GC B cells among B220<sup>+</sup> B cells in the draining LNs on day 2 post-immunization with TNP-KLH were comparable in *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice and controls (Supplemental Figure 2 and Figure 2G). To fully examine pre-GC development, use of hapten specific B cells would be required. Furthermore, the proliferation of pre-GC B cells, as indicated by the MFI of Ki-67 staining, was also similar between the two groups (Figure 2G). These results suggest that impaired generation of GC B cells in *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice is not secondary to defective T-B cell interactions in the interfollicular area of the LN.

**DOCK8 expression in T cells is dispensable for the generation of Tfh cells and their *in vitro* function.** Given the reduced number of GC B cells in *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice immunized with TNP-KLH (Figure 2C), we examined Tfh cells in draining LNs 7 days after immunization. The percentages and numbers of CXCR5<sup>+</sup>PD1<sup>+</sup> Tfh cells were similar in *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice and controls (Figure 3A). T follicular regulatory (Tfr) cells express Tfh markers as well as FOXP3 and downregulate the GC reaction (30). The Tfr:Tfh cell ratio in draining LNs was also comparable in immunized *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice and controls (Figure 3B).

We next investigated whether DOCK8 deficient Tfh cells have an altered phenotype that may impair their function. The Tfh cell markers CXCR5, PD1, and ICOS not only help define the Tfh cell population, but are important for Tfh cell recruitment to the follicle, expression of BCL6, and survival (3-6, 31-33). Surface expression of CXCR5 and PD1 was normal, but ICOS expression was slightly decreased in Tfh cells from *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>*

mice compared to controls (Figure 3C). Intracellular expression of BCL6 in Tfh cells was similar between the two groups (Figure 3D). Tfh cells highly express CD40L and SLAM family members (34-36). CD40L is important for the generation of GC reactions (37), while SLAM (CD150) is crucial for cytokine production by Tfh cells (36). Moreover, the SLAM family member, CD84, plays a critical role stabilizing Tfh-B cell interactions (35, 36) and the SLAM-associated adapter protein (SAP) is required for late stage Tfh cell differentiation, Tfh-B cell conjugate formation, and GC development (35, 38-40). Surface expression of CD40L, the SLAM molecules CD150 and CD84, as well as mRNA levels of *Sh2d1a* (encoding SAP) were comparable in Tfh cells from the two groups (Figure 3E). Furthermore, intracellular staining revealed similar percentages of IL-4 and IL-21 expressing Tfh cells in *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice and controls (Supplemental Figure 3A and Figure 3F).

We conducted an unbiased screen for differences in gene expression between DOCK8 deficient and WT Tfh cells. We used a >1.5 fold change with a significance of  $p < 0.05$  as the threshold for a significant change in gene expression. RNAseq analysis revealed that expression of 18 genes was significantly downregulated in Tfh cells from *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice compared to controls. They included genes encoding proteins involved in cell-cell interactions - CD96, CD73 (*Nt5e*), *Sirpa*, *Cldn25*, and *Ifitm3* (41-45), and in cell motility and shape - *Myo1e*, *Actg2*, and *Klhl4* (46-48) (Supplemental Figure 3B). There was also reduced expression of genes encoding the chemokine CCL8, ceramide synthase 4, and galectin like protein (*Lgalsl*) (49, 50). Only 4 genes were expressed at a higher level in Tfh cells from *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice. They included *B4galt4*, *Bckdhb*, *Tbc1d23*, and *Timmdc1*, all encoding intracellular proteins with

unknown relevance to Tfh cell function (Supplemental Figure 3B). Notably, there were no significant differences in the expression of *Il4*, *Il13*, *Il5*, or *Il21* between Tfh cells from *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice and controls (Supplemental Figure 3B). Decreased expression of genes important for cell-cell interaction and cell motility in Tfh cells may have contributed to defective GC formation in *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice.

Conjugate formation between T cells and B cells presenting cognate antigen allows these B cells to effectively compete for cytokines crucial for their differentiation (51). The ability of DOCK8 deficient T cells to form conjugates with WT B cells *in vitro* was examined. OVA peptide specific CD4<sup>+</sup> T cells were purified from *Dock8<sup>-/-</sup>* OTII mice and OTII controls and incubated for 3 hours with LPS activated WT B cells pulsed with OVA<sub>329-337</sub> peptide. Conjugate formation was assessed by flow cytometry. DOCK8 deficient CD4<sup>+</sup> T cells and WT CD4<sup>+</sup> T cells had similar percentages of conjugate formation with WT B cells loaded with OVA peptide (Figure 3G). Similarly, DOCK8 deficient CD4<sup>+</sup> T cells had intact conjugate formation with WT bone marrow derived DCs (BMDCs) pulsed with OVA peptide (Supplemental Figure 3C). Thus, in the absence of spatial constraints, short term *in vitro* conjugate formation between DOCK8 deficient T cells and APCs (both B cells and DCs) is intact.

Next, the ability of DOCK8 deficient Tfh cells to promote *in vitro* B cell differentiation was examined. WT and DOCK8 deficient CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>CD25<sup>-</sup>CD19<sup>-</sup> Tfh cells were sorted from the draining LN of TNP-KLH immunized mice and cultured with WT CD19<sup>+</sup> B cells in the presence of anti-CD3 and anti-IgM. After 6 days in culture, B cell surface markers were examined by flow cytometry. WT and DOCK8 deficient Tfh cells were comparably potent in inducing the expression of GL7 and the glucose transporter GLUT1

on B cells (Figure 3H). In addition, a comparable percentage of B cells were sIgG1<sup>+</sup> after stimulation with DOCK8 deficient Tfh cells and WT Tfh cells (Figure 3H). These data demonstrate that DOCK8 deficient Tfh cells are normal in numbers, ability to secrete cytokines, and provide help for the generation of GC B cells *in vitro*.

**DOCK8 deficient T cells localize poorly to the GC, have impaired adhesion to ICAM-1, and fail to activate LFA-1 following TCR/CD3 ligation.** Tfh cell migration into the B cell follicle results in GC formation (1). GCs from the draining LNs of immunized *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice had fewer T cells than those from control mice (Supplemental Figure 4A). This prompted us to investigate whether DOCK8 deficient Tfh cells properly localize within the GC. CD4<sup>+</sup> T cells from CD45.2<sup>+</sup> *Dock8<sup>-/-</sup>* OTII mice or OTII controls were adoptively transferred into CD45.1<sup>+</sup> recipients. Recipients were immunized the day after in the hock with NP-OVA, and draining LNs were examined nine days later. The GC area was comparable in recipients of DOCK8 deficient T cells and recipients of WT T cells (Supplemental Figure 4B), and endogenous CD45.1<sup>+</sup> T cells made up 59-62% of the Tfh cells that developed regardless of whether DOCK8 deficient or WT CD45.2<sup>+</sup> T cells were transferred. This reflects the intact ability of the WT recipients to mount a GC reaction. Comparable numbers of CD45.2<sup>+</sup> T cells from *Dock8<sup>-/-</sup>* donors and WT donors were found in the recipients' LNs (Figure 4A). The percentage of CD45.2<sup>+</sup> donor cells that developed into Tfh cells was comparable (Supplemental Figure 4C). In contrast, the percentage of donor CD45.2<sup>+</sup> T cells in the follicle that localized to the GC was significantly reduced in recipients of *Dock8<sup>-/-</sup>* T cells compared to control T cells (Figure 4B). Our results suggest that antigen activated DOCK8 deficient T cells have impaired ability to migrate into the GC.

Tfh cells migrate towards CXCL13 produced by stromal cells in B cell follicles (6). Expression of the CXCL13 receptor, CXCR5, was comparable in Tfh cells from *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice and controls (Figure 3C). To examine the ability of Tfh cells to migrate in response to CXCL13, *Dock8<sup>-/-</sup>* OTII and WT OTII mice were immunized in the hocks and *i.p.* with NP-OVA in alum to generate CXCR5<sup>+</sup> CD4<sup>+</sup> T cells. Ten days after immunization, CD4<sup>+</sup> T cells were isolated from the spleen and draining LNs. Approximately 40-45% of the cells in the CD4<sup>+</sup> T cell preparation were CXCR5<sup>+</sup> from both *Dock8<sup>-/-</sup>* and control mice. We found no significant difference in the ability of *Dock8<sup>-/-</sup>* and control CD4<sup>+</sup> T cells to migrate in response to CXCL13 in a transwell assay (Supplemental Figure 4D). This suggests that *Dock8<sup>-/-</sup>* T cells have an intact CXCR5-CXCL13 axis.

Interaction between LFA-1, highly expressed on Tfh cells, and ICAM-1/2 on B cells is important for Tfh cell migration into GCs (7, 9). Following TCR ligation, LFA-1 undergoes a rapid conformational change to its activated form that displays high affinity for ICAMs (52, 53). LFA-1 surface expression was comparable in WT and DOCK8 deficient murine CD4<sup>+</sup> T cells (Figure 4C). However, anti-CD3 activated CD4<sup>+</sup> T cells from *Dock8<sup>-/-</sup>* mice demonstrated significantly decreased binding to immobilized ICAM-1-Fc under physiologic flow conditions (Figure 4D). Following stimulation with PMA, which bypasses the TCR, adhesion to ICAM-1 was comparable in DOCK8 deficient and WT CD4<sup>+</sup> T cells (Figure 4D).

Using a monoclonal antibody (mAb m24) that selectively recognizes the active conformation of LFA-1 on human cells, we examined the ability of T cells from DOCK8 deficient patients with homozygous *DOCK8* mutations that abrogated protein expression (see Materials and Methods) to activate LFA-1 following TCR/CD3 ligation. Surface expression of LFA-1 was comparable in unstimulated CD4<sup>+</sup> T cells from the patients and

controls and remained unchanged after 30 minutes of anti-CD3 stimulation (Figure 4E). Following TCR/CD3 ligation, CD4<sup>+</sup> T cells from controls demonstrated a significant increase in activated LFA-1 on their surface (Figure 4F). Activated CD4<sup>+</sup> T cells from patients demonstrated a small increase in activated LFA-1 that was significantly less than in controls (Figure 4F). These results suggest that decreased T cell LFA-1 activation, leading to a defective interaction with ICAM/2 on B cells, may impair migration of DOCK8 deficient Tfh cells into GCs.

## DISCUSSION

We demonstrate that DOCK8 expression in T cells is critical for the migration of Tfh cells into GCs and thereby for normal GC formation, generation of GC B cells, and production of high affinity IgG antibodies to TD antigens.

Recently, Gowthaman *et al.* reported that *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice immunized with NP-OVA plus LPS intranasally demonstrate reduced IgG antibody levels with decreased affinity. However, in contrast to our findings, serum IgE levels were elevated, GCs were normal in size, and GC B cells were not reduced (54). Immunizing our *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice using the same protocol yielded similar results (data not shown). LPS is an adjuvant that activates B cells and innate cells via TLR4 and promotes Th1 responses at high doses and Th2 responses at low doses (55, 56). In contrast, alum, the adjuvant we used, is commonly used in human vaccines and promotes type 2 cytokine production by innate cells including ILC2s and NKT cells (57-60). Differences in the mechanisms of action of these adjuvants, as well as the route of immunization, may underlie the differences between the study of Gowthaman *et al.* and ours.

Although required for normal GC formation, DOCK8 expression in T cells was not required for the generation of Tfh cells. DOCK8 expression also was not required for Tfh cells to express cytokines and surface molecules important for GC formation, the formation of antigen dependent conjugates with B cells or DCs, or *in vitro* Tfh driven B cell differentiation into GC B cells. The primary defect observed in DOCK8 deficient Tfh cells was their reduced capacity to migrate into GCs. DOCK8 deficiency may affect signaling downstream of chemoattractants. In addition, normal expression of SLAM family members by DOCK8 deficient T cells does not rule out defective signaling by these molecules, which

would contribute to the defective migration of these cells into GCs and impaired generation of GC B cells.

A major finding of our study is that DOCK8 is essential for activation of LFA-1 following TCR ligation and for the ability of TCR activated T cells to bind to ICAM-1. Since the interaction of LFA-1 on Tfh cells with ICAM-1/2 on B cells is important for GC formation (61), defective LFA-1 activation in T cells likely contributes to the defective GC formation in DOCK8 deficiency. Diminished binding to ICAM-1 by activated DOCK8 deficient total T cells, CD8<sup>+</sup> T cells, Treg cells, and B cells has been previously noted (20, 62-64).

DOCK8 exists in a macromolecular complex with WASP and Talin (63, 65). Downstream of antigen receptor engagement, DOCK8 loads CDC42 with GTP, leading to WASP activation (63). WASP activation is important for LFA-1 polarization and function (66, 67). TCR ligation, also causes the DOCK8 associated protein Talin to bind to the  $\beta$  chain of integrins, which is critical for LFA-1 activation (65, 68). Either LFA-1 blockade or deletion of Talin-1 blocks the generation of Tfh cells (69). Thus, both WASP and Talin may mediate DOCK8 dependent LFA-1 activation following TCR

It is noteworthy that DOCK8 deficiency in T cells affects the generation of GC B cells, but not of Tfh cells. The residual LFA-1 activation and adhesion to ICAM-1 expressing cells in antigen activated DOCK8 deficient T cells may allow sufficient contact between T and B cells to generate normal numbers of Tfh cells, but may not be sufficiently strong and/or long-lasting to allow normal Tfh cell migration into GCs. Impaired migration of Tfh cells into GCs results in compromised GC B cell development and defective IgG antibody production and affinity maturation in DOCK8 deficiency.

## METHODS

*Study approval.* All mouse studies were approved and performed in accordance with Boston Children's Hospital Institutional Animal Research and Care Committee. All human participants gave written informed consent to participate in a research protocol approved by the Committee on Clinical Investigation at Boston Children's Hospital.

*Mice.* *Dock8*<sup>-/-</sup> and *Dock8*<sup>flox/flox</sup> mice are described in references (62, 63). *Dock8*<sup>flox/flox</sup> mice were mated with *Cd4-Cre*<sup>Tg</sup> mice (Taconic) to generate *Cd4-Cre*<sup>Tg</sup>*Dock8*<sup>flox/flox</sup>. *Dock8*<sup>-/-</sup> mice were bred with OTII mice (Charles River) to generate *Dock8*<sup>-/-</sup> OTII mice. Both female and male mice were studied. All mice were kept in a specific pathogen free environment.

*Patients.* Blood samples were obtained from healthy volunteers and four DOCK8 deficient patients with homozygous mutations in *DOCK8* (c.3191delA, c.4826\_4827delAC, and c.3787delA) that abolish DOCK8 expression.

*Immunoblotting.* Total B cells and T cells were purified from the spleens of *Cd4-Cre*<sup>Tg</sup>*Dock8*<sup>flox/flox</sup> and controls using magnetic beads (Miltenyi). Cells were lysed in 1% Triton buffer (150 mM NaCl, 25 mM Tris-Cl, pH 7.5, 5 mM EDTA) containing complete protease inhibitors (Roche). Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the mAbs against DOCK8 (H159, Santa Cruz) and  $\beta$ -actin (ab8226, abcam).

*Immunizations.* Mice were immunized either *i.p.* or in the bilateral hocks with 10  $\mu$ g of TNP-KLH (Biosearch, T-5060), NP-OVA (Biosearch, N-5051), or OVA (Sigma-Aldrich) in alhydrogel adjuvant (vac-alu-250, Invivogen), and boosted at day 14 with 2.5  $\mu$ g of antigen in adjuvant. Sera were collected at days 0, 14, and 21. To study pre-GC B cell, GC

B cell, Tfh cell generation, or GCs, draining LNs (inguinal and popliteal) were harvested on days 2, 7, or 10 from hock immunized mice. To examine antibody affinity maturation, mice were immunized in the bilateral hocks with 1 µg of TNP-KLH, and sera was collected on day 14.

*ELISAs.* For antigen-specific antibody responses, 96-well plates (ThermoFisher) were coated overnight at 4°C with 10 µg/ml TNP<sub>4</sub>-BSA (Biosearch), TNP<sub>38</sub>-BSA (Biosearch), NP-BSA (Biosearch), or OVA (Sigma-Aldrich). Blocking was accomplished with 3% BSA in PBS. Serial dilutions of sera were applied to the plates overnight at 4°C. After washing, AP-conjugated goat anti-mouse IgG (Southern Biotech) was applied to the plates for 2 h at room temperature. Bound secondary antibodies were detected by incubation with p-nitrophenyl phosphate (PNPP) substrate (Sigma-Aldrich). For detection of total serum IgE, 96-well plates were coated with anti-IgE (R35-72, BD Biosciences). For detection of total and TNP-specific IgE antibodies, biotin-conjugated rat anti-mouse IgE (R35-118, BD Biosciences) was applied to plates for 2 h at room temperature. Bound secondary antibodies were detected with avidin-conjugated HRP (ThermoFisher) and developed by incubation with TMB substrate (ThermoFisher).

*Cellular proliferation and cytokine secretion.* Inguinal and popliteal LNs were harvested at day 21 from mice immunized in the hock with TNP-KLH. Cells were stained with Cell Trace Violet (Life Technologies) and stimulated with the indicated concentrations of KLH antigen (374825, Calbiochem). After 24 h and 72 h in culture, IL-2 and IFN-γ contents were measured by ELISA according to the manufacturer's instructions (ThermoFisher). Proliferation was assessed by flow cytometry on day 5 of culture.

*Flow cytometry.* Single cell suspensions were made of the draining LNs. For staining of surface molecules, cells were incubated on ice with fluorochrome conjugated mAbs against CD3 (145-2c11), CD4 (GK1.5), CD8 (53-6.7), GL7 (GL7), FAS (15A7), IgD (11-26c.2a), CCR6 (29-2L17), PD1 (29F.1A12), ICOS (C398.4A), CD25 (PC61.5), CD40L (MR1), CD84 (mCD84.7), SLAM (TC15-12F12.2), CD19 (6D5) from Biolegend, B220 (RA3-6B2) from ThermoFisher and GLUT1 (EPR3915) from Abcam. For CXCR5 staining, biotinylated CXCR5 (SPRLC5, ThermoFisher) was used followed by streptavidin-PE or streptavidin-PE Texas Red (Biolegend). For intracellular staining with antibodies against FOXP3 (FJK-165), KI-67 (16A8), BCL6 (K112-91) and IgG1 (A85-1) from Biolegend, cells were fixed, permeabilized, and stained according to the manufacturer's directions (ThermoFisher).

For intracellular cytokine staining, cells were stimulated with phorbol 12,13-dibutyrate and ionomycin (Sigma Aldrich) in the presence of Golgistop and Golgiplug (BD Biosciences) for 4 hours. Cells were then fixed and permeabilized using a BD Bioscience Cytotfix/Cytoperm kit and incubated with a fluorochrome conjugated antibody against IL-4 (11B11, ThermoFisher) and with recombinant human IL-21R Fc chimera protein (991-R2-100, R&D systems) followed by Alexa Fluor 647 AffiniPure F(ab')<sub>2</sub> Fragment Goat Anti-Human IgG (109-606-008, Jackson Immunoresearch).

*LFA-1 expression on human T cells.* Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Paque PLUS density gradient centrifugation (GE Healthcare). PBMCs were stimulated by incubation with anti-CD3 (OKT3, ThermoFisher) followed by cross-linking with goat anti-mouse IgG (115-006-072,

Jackson Immunoresearch). Samples were stained with total LFA-1 (TS2/4), active LFA-1 (m24), CD4 (OKT4), and Zombie Violet viability dye all from Biolegend.

All data was acquired on a BD LSRIIFortessa cell analyzer using FACSDiva software (BD Biosciences). Analyses were performed using FlowJo software (Tree Star, Inc.).

*Bone marrow derived DC generation.* DCs were generated from WT mice. The femurs and tibias were isolated. BM cells were flushed from the marrow cavities. Red blood cells were lysed with ACK buffer. Bone marrow cells were cultured in media supplemented with 10 ng/ml GM-CSF and 10 ng/ml IL-4. After 6 days, the immature DCs were harvested and re-plated with 50 ng/ml LPS. Cells were harvested 24 later for use in *in vitro* conjugation assays.

*In vitro Conjugation Assay.* CD4<sup>+</sup> OT-II T cells were isolated from the spleens of *Dock8*<sup>-/-</sup> OTII mice or OTII controls using the Miltenyi CD4<sup>+</sup> T cell isolation kit and stained with anti-mouse CD4-APC-Cy7 (RM45, Biolegend). B cells were isolated from WT spleens using magnetic beads (Miltenyi) and stimulated with 1 µg/mL LPS plus OVA<sub>323-339</sub> peptide (AnaSpec) for 3 hours, washed, and then stained with anti-mouse CD19 BV605 (6D5, Biolegend). OTII CD4<sup>+</sup> T cells (5×10<sup>5</sup>/well) and activated B cells (2×10<sup>6</sup>/well) were incubated for 3 h at 37°C in 96 well U-bottom plates. To examine CD4<sup>+</sup> T cell conjugate formation with BMDCs, BMDCs were stimulated for 3 hours with OVA<sub>323-339</sub> peptide. OTII CD4<sup>+</sup> T cells (2×10<sup>5</sup>/well) and BMDCs (1×10<sup>5</sup>/well) were incubated for 3 h at 37°C in 96 well U-bottom plates. Conjugate frequencies were enumerated by flow cytometry.

*In Vitro Stimulation Assays.* For *in vitro* B cell stimulation assays with Tfh cells, *Cd4-Cre*<sup>Tg</sup>*Dock8*<sup>flox/flox</sup> mice and controls were immunized with TNP-KLH in alhydrogel in the hock. 7 days later, the popliteal and inguinal LNs were harvested.

CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>CD25<sup>-</sup>CD19<sup>-</sup> Tfh cells were isolated by cell sorting. 3x10<sup>4</sup> WT or DOCK8 deficient Tfh cells were plated with 5x10<sup>4</sup> B cells (sorted as CD19<sup>+</sup>CD4<sup>-</sup> cells from lymph nodes of TNP-KLH immunized *Cd4-Cre<sup>Tg</sup>* controls) along with 2 µg/ml soluble anti-CD3 (BioExcell) and 5 µg/ml anti-IgM (Jackson ImmunoResearch). Assays were performed as described as previously described (70). Six days later, cell surface markers were analyzed by flow cytometry (described above).

*Quantitative analysis of gene expression.* Tfh cells were isolated by fluorescent sorting for CD4<sup>+</sup>CD19<sup>-</sup>CD25<sup>-</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup> cells from the draining LNs of mice immunized in the hock with TNP-KLH. RNA was isolated using an RNeasy Plus micro kit (Qiagen) according to the manufacturer's instructions.

Complementary DNA was reverse transcribed using an iScript cDNA synthesis kit (Biorad) according to the manufacturer's instructions. Analysis of transcripts was performed with a commercial Taqman primer/probe set against mouse *Sh2d1a* with *B2m* as a control (ThermoFisher). Relative mRNA expression was quantified using the 2<sup>-ΔΔC<sub>t</sub></sup> method.

For RNAseq, RNA was isolated as described above from Tfh cells. Low input mRNA libraries (Clontech SMARTer v4) were generated and sequenced on an Illumina NS500 Single-End 75bp (SE75) by the Dana Farber Cancer Institute Molecular Biology Core Facility. TopHat was used to align reads to mouse genome [Mm9, National Center for Biotechnology Information (NCBI)], and HTSeq was used to estimate read counts. DESeq2 was used to normalize data and assess differential gene expression with a *p* < 0.05. Expression was normalized using the geometric mean for each gene. Heat maps were generated using Prism (GraphPad Software, Inc.) and data is shown as log<sub>2</sub> fold change relative to the geometric mean.

*LN Imaging.* LNs from immunized mice were frozen in Tissue-Tek OCT Compound and cryosectioned. Sections were fixed with 4% PFA for 10 min, washed three times in PBS, blocked 45 minutes with 10% goat serum in PBS, and stained 1 hour with 1:100 antibodies in 2% BSA in PBS. For GC imaging, fluorochrome conjugated antibodies against TCR $\beta$  (H57-597), IgD-FITC (11.26c.2), and GL7 (GL7) from Biolegend were utilized. After washing 3 times with PBS, stained sections were mounted with ProLong Diamond Antifade Mountant (ThermoFisher). Images were acquired on a Zeiss Elyra PS.1 confocal microscope and analyzed in Imaris 8.4.1.

For adoptive transfer studies, CD4<sup>+</sup> T cells were isolated from the spleens and LNs of OTII and *Dock8*<sup>-/-</sup>OTII mice using magnetic beads (Miltenyi). 10x10<sup>6</sup> cells were injected *i.v.* into CD45.1<sup>+</sup> mice. 24 h later, recipient CD45.1<sup>+</sup> mice were immunized in the hock with NP-OVA. After 9 days, the popliteal LNs were harvested and processed as described above. In addition to the antibodies noted above, fluorochrome conjugated antibodies to CD3 (17A2) and CD45.2 (104) from Biolegend were used. Images were acquired using a Zeiss LSM 880 Upright Laser Scanning Confocal Microscope and analyzed using Image J. For quantification, CD45.2<sup>+</sup> cells were counted in the GC and surrounding follicle, and the percentages of CD45.2<sup>+</sup> T cells in the GCs out of total CD45.2<sup>+</sup> cells in the GC+follicle were calculated. Data was normalized by the area of GCs and follicles as described in ref (71).

*Transwell migration to CXCL13.* CD4<sup>+</sup> T cells were isolated from the spleens and draining LNs of OTII and *Dock8*<sup>-/-</sup>OTII mice immunized *i.p.* and in the hock with NP-OVA+alum using magnetic beads (Miltenyi). 1x10<sup>5</sup> CD4<sup>+</sup> T cells were applied to the upper chambers of a 5  $\mu$ m pore 96-well plate. CXCL13 was added to the bottom chamber in media with 1% FCS at concentrations of 0, 500, and 1000 ng/ml.

*T cell adhesion to immobilized ICAM1 under laminar shear flow conditions.* Live-cell imaging of CD4<sup>+</sup> T cells adhesion was recorded by a video camera coupled to a Nikon TE2000 inverted microscope equipped with a 20x/0.75 NA phase contrast objective and VideoLab software (Mitov) (72, 73). *Dock8*<sup>-/-</sup> and WT CD4<sup>+</sup> T cells were activated with anti-CD3 cross-linking or PMA for 20 min. Cells ( $5 \times 10^5$  cells were suspended in 100  $\mu$ l PBS) were flowed across immobilized 20  $\mu$ g/ml ICAM-1-Fc in the presence of 250 ng/ml SDF-1 $\alpha$  at a shear stress of 0.75 dynes/cm<sup>2</sup> (73). CD4<sup>+</sup> cell adhesion was determined in 5 separate fields for 1 min and reflects accumulation of adherent cells.

*Statistical Analysis.* Comparisons were analyzed for statistical significance using unpaired two tailed Student's *t* test, one-way ANOVA with Tukey's multiple comparison tests, and two-way ANOVA to determine the p value using Prism software (GraphPad Software, Inc.). A p value of <0.05 was considered significant. Germinal center size responses were fit to a linear mixed-effects model in R (version 3.4.2) and tested using an analysis of variance (ANOVA). Genotype was specified as a fixed effect; immunization batch, individual, and lymph node were treated as nested random effects.

## **AUTHOR CONTRIBUTIONS**

E.J., M.T., J.B., S.G., P.T.S., F.V., C.K., E.M., and M.D. performed the experiments and analyzed the data. A.S. and S.A. provided patients samples. E.J, F.W.L, F.B., and R.S.G designed and supervised the research. E.J., M.T., and R.S.G wrote the manuscript.

## **ACKNOWLEDGEMENTS**

We thank Zach Herbert from the Molecular Biology Core Facilities at Dana-Farber Cancer Institute for library preparation and sample sequencing, and Drs. Joe Craft, Jason Weinstein, and Michael Carroll for their help and advice. This research was supported by K08AI114968 (E.J.), RO1AI114588 (R.S.G.) and the Perkin Fund (R.S.G.). The authors declare no competing financial interest.

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## Figure Legends

### **Figure 1. Impaired antibody responses to TD antigens in *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice.**

**A.** TNP-specific serum IgG levels on day 0 (unimmunized) and day 21 (immunized) from *Dock8<sup>-/-</sup>* (left), *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* (right), and controls after immunization in the bilateral hocks with TNP-KLH. **B.** NP-specific serum IgG (left), TNP-specific serum IgG (middle), and OVA-specific serum IgG (right) measured on day 0 and day 21 after immunization of *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice and controls with NP-OVA in the hocks, TNP-KLH *i.p.*, or OVA *i.p.* A and B show data from one representative experiment out of two. n=4-5 mice/group. Data are presented as mean±SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 by ANOVA.

### **Figure 2. *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice have a marked reduction in GC B cells after immunization with TD antigen.**

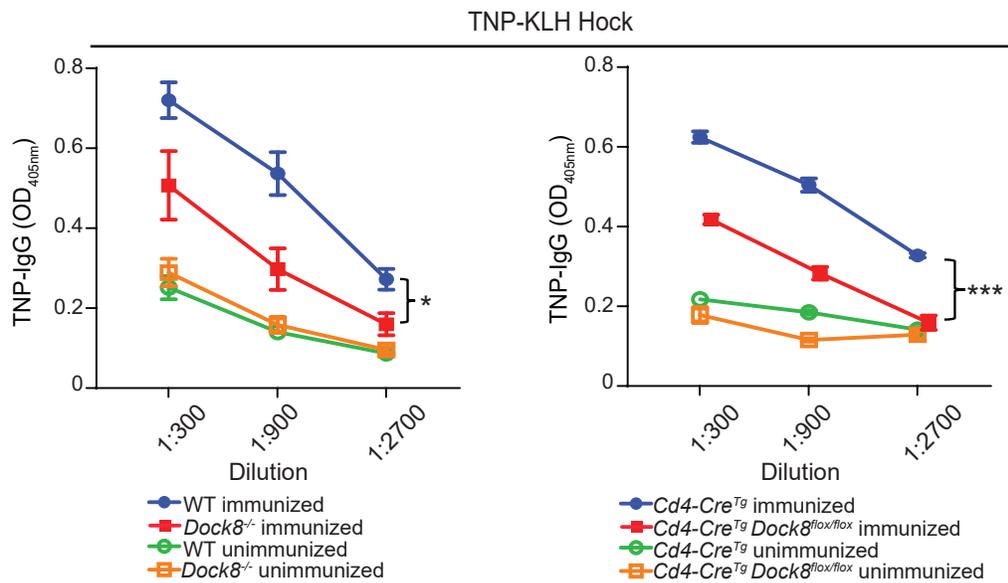
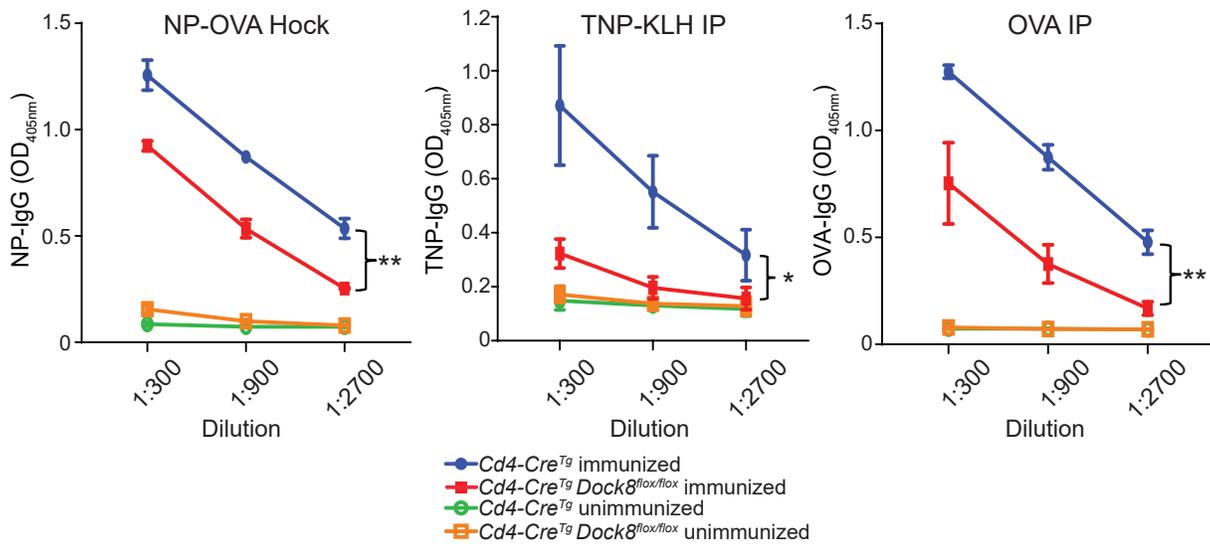
Draining LNs from *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* and control mice immunized in the hocks with TNP-KLH were examined on day 2 for pre-GC B cells, on day 7 for GC B cells, and on day 10 for GC size. **A.** Representative immunofluorescence photomicrograph of popliteal LNs (left). B cell follicles (IgD<sup>+</sup>) are green, GCs (GL7<sup>+</sup>) are red, and the T cell zones (TCR $\alpha$ <sup>+</sup>) are blue. Images are at 20x magnification. Quantification of GC size (right). n=4-5 mice/group in 2 pooled independent experiments. ANOVA: \*p<0.05. **B.** TNP-specific IgG affinity collected on day 14 after immunization. n=4 mice/group. Results are representative of 2 independent experiments. **C.** Representative flow cytometry plots (left), percentages (middle), and numbers (right) of FAS<sup>+</sup>GL7<sup>+</sup> GC B cells in the draining LNs. N=7 mice/group. **D.** MFI of surface FAS (left) and GL7 (right) by B220<sup>+</sup>GL7<sup>+</sup> and B220<sup>+</sup> FAS<sup>+</sup> cells, respectively. N=5-6 mice/group. **E.** Percentages of BCL6<sup>+</sup>KI-67<sup>+</sup> B cells in the draining LNs. N=4 mice/group. **F.** MFI of BCL6 (left) and KI-67

(right) in BCL6<sup>+</sup> B cells. N=4 mice/group. **G.** Percentage of FAS<sup>int</sup>GL7<sup>lo</sup>IgD<sup>hi</sup>CCR6<sup>hi</sup> pre-GC B cells (left) and MFI of KI-67 in pre-GC B cells (right). n=7 mice/group from 2 pooled experiments. D-F show a representative experiment out of 3. Data are presented as mean±SEM. t-test \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

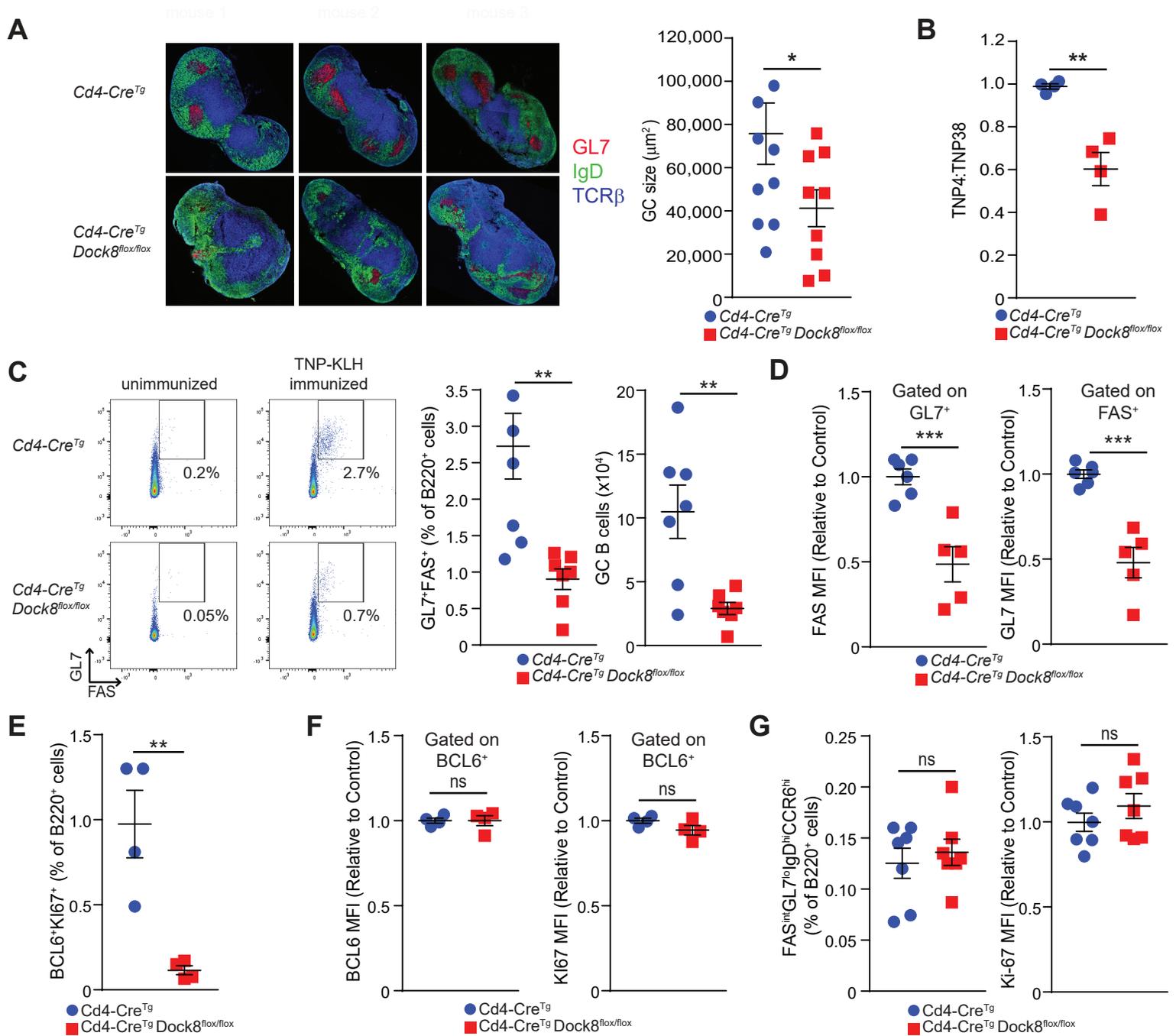
**Figure 3. *Cd4-Cre<sup>Tg</sup>Dock8<sup>fllox/fllox</sup>* mice have a normal Tfh percentage and phenotype after immunization with TD antigen.** *Cd4-Cre<sup>Tg</sup>Dock8<sup>fllox/fllox</sup>* mice and controls were immunized with TNP-KLH in the hock. Draining LNs were analyzed 7 days after immunization. **A.** Representative FACS plots (left), percentages (middle) and numbers of CXCR5<sup>+</sup>PD1<sup>+</sup> Tfh cells (right). Pooled results from 3 individual experiments, n=9 mice/group. **B.** Ratio of Tfr:Tfh cells. **C.** MFI of CXCR5, PD1, and ICOS expression by Tfh cells. **D.** Intracellular BCL6 expression by Tfh cells. **E.** MFI of CD150, CD84, and CD40L expression by Tfh cells (left). qPCR analysis of *Sh2d1a* mRNA expression in sorted Tfh cells (right). Results are expressed as fold increase of the *Sh2d1a* mRNA:  $\square$  2 *microglobulin* mRNA ratio relative to control. **F.** Intracellular expression of IL-4 and IL-21 by Tfh cells stimulated for 4 hours with phorbol-12,13-dibutyrate and ionomycin. **G.** Percentage of T-B cell conjugates out of total CD4<sup>+</sup> T cells isolated from *Dock8<sup>-/-</sup>* OTII and WT OTII mice incubated for 3 h with LPS stimulated WT B cells pulsed with OVA<sub>323-339</sub>. **H.** Sorted CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>CD25<sup>-</sup>CD19<sup>-</sup> Tfh cells from the draining LNs were incubated with CD19<sup>+</sup> B cells sorted from the LNs of WT mice in the presence of soluble anti-CD3 and anti-IgM. Cell surface expression of GL7, GLUT1, and IgG1 by B cells after 6 days in culture. Results in B-H are representative of 3 independent experiments. Data are presented as mean±SEM. t-test \*p<0.05

**Figure 4. DOCK8 deficient CD4<sup>+</sup> T cells have decreased migration into GCs and impaired activation of LFA-1. A, B.** CD4<sup>+</sup>CD45.2<sup>+</sup>OT-II WT or DOCK8-deficient T cells were adoptively transferred into CD45.1<sup>+</sup> WT mice. Recipients were immunized with NP-OVA in the hock, and popliteal LNs were analyzed on day 9 post-immunization. (A) The number of OTII CD45.2<sup>+</sup> T cells found in the draining LNs of recipient mice were analyzed by flow cytometry. (B) The localization within the draining LNs of transferred CD4<sup>+</sup>CD45.2<sup>+</sup>OT-II WT or DOCK8-deficient T cells were examined by immunofluorescence microscopy. Representative photomicrographs at 20x magnification (left). B cell follicles (IgD<sup>+</sup>) are green, GCs (GL7<sup>+</sup>) are red, CD45.2<sup>+</sup> T cells are white, and the T cell zones (CD3<sup>+</sup>) are blue. Percentage of CD45.2<sup>+</sup> in GCs relative to the total CD45.2<sup>+</sup> cells in GC plus the surrounding follicle normalized to the GC and follicle area (right). n=5 mice/group. **C.** MFI of total surface LFA-1 expression on CD4<sup>+</sup> T cells from *Dock8*<sup>-/-</sup> mice and WT controls. **D.** Number of adherent CD4<sup>+</sup> T cells, stimulated with anti-CD3 or PMA from *Dock8*<sup>-/-</sup> mice and controls to ICAM-1, 10 min after application to a flow chamber with a flow rate of 0.75 dynes/cm<sup>2</sup>. Five fields were examined per slide. **E.** Representative histograms (left) and MFI of LFA-1 surface expression (right) by unstimulated and anti-CD3 stimulated CD4<sup>+</sup> T cells from DOCK8 deficient patients and healthy shipping controls (n=3/group). MFI values were normalized to the mean value of shipping controls. **F.** Representative histograms (left) and MFI of activated LFA-1 expression (right) by unstimulated and anti-CD3 stimulated CD4<sup>+</sup> T cells from DOCK8 deficient patients and healthy shipping controls (n=3/group). MFI values were normalized to the mean value of unstimulated shipping controls. Results in C and D are of 3

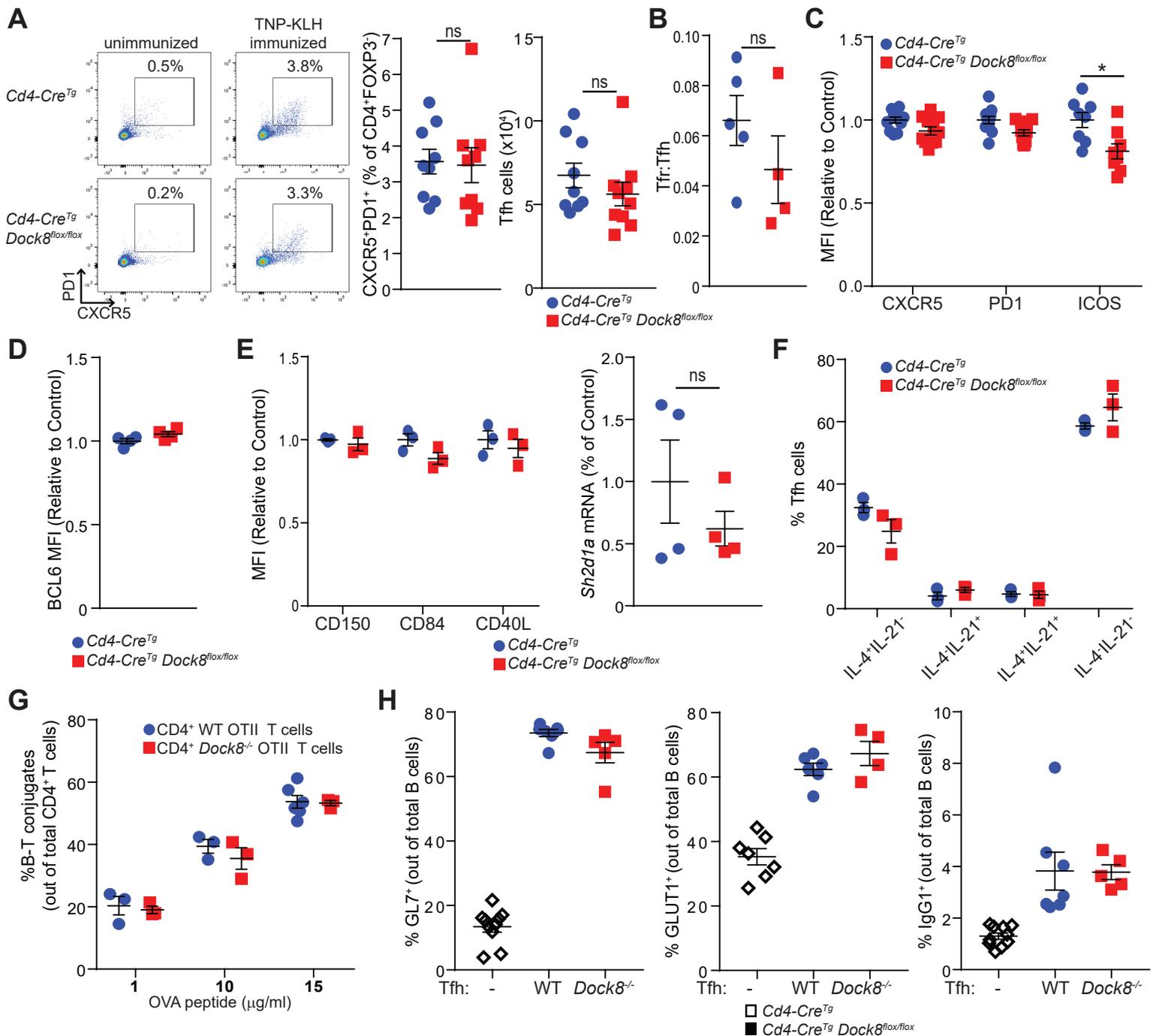
independent experiments each with 3 mice/group. Data in A-F are presented as mean $\pm$ SEM. In A-C, t-test \* $p$ <0.05. In D-E, one-way ANOVA with Tukey's multiple comparisons \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001.

**A****B**

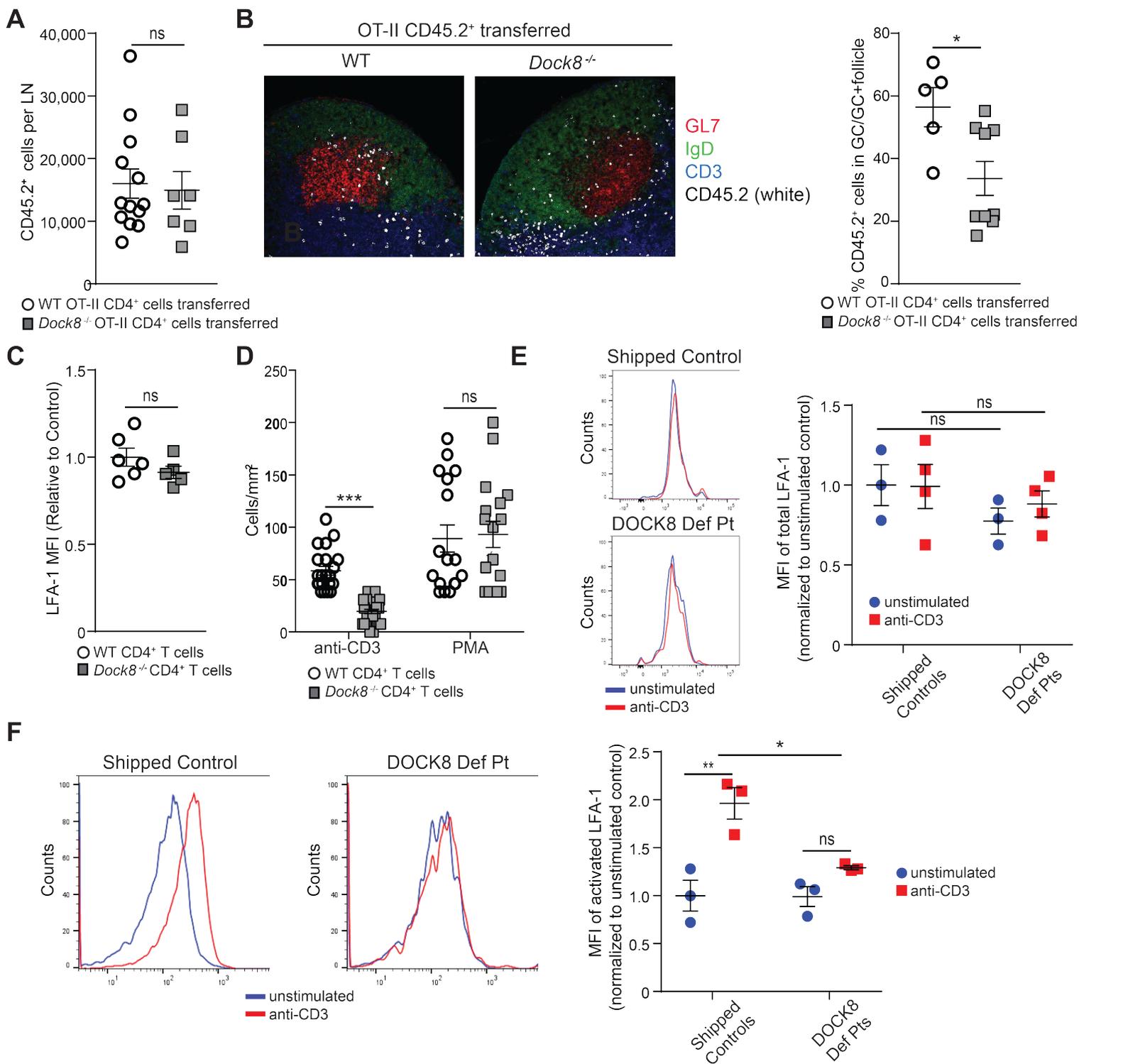
**Figure 1. Impaired antibody responses to TD antigens in *Cd4-Cre*<sup>Tg</sup>*Dock8*<sup>flox/flox</sup> mice.** **A.** TNP-specific serum IgG levels on day 0 (unimmunized) and day 21 (immunized) from *Dock8*<sup>-/-</sup> (left), *Cd4-Cre*<sup>Tg</sup>*Dock8*<sup>flox/flox</sup> (right), and controls after immunization in the bilateral hocks with TNP-KLH. **B.** NP-specific serum IgG (left), TNP-specific serum IgG (middle), and OVA-specific serum IgG (right) measured on day 0 and day 21 after immunization of *Cd4-Cre*<sup>Tg</sup>*Dock8*<sup>flox/flox</sup> mice and controls with NP-OVA in the hocks, TNP-KLH i.p., or OVA i.p. A and B show data from one representative experiment out of two. n=4-5 mice/group. Data are presented as mean±SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 by ANOVA.



**Figure 2. *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice have a marked reduction in GC B cells after immunization with TD antigen.** Draining LNs from *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* and control mice immunized in the hocks with TNP-KLH were examined on day 2 for pre-GC B cells, on day 7 for GC B cells, and on day 10 for GC size. **A.** Representative immunofluorescence photomicrograph of popliteal LNs (left). B cell follicles (IgD<sup>+</sup>) are green, GCs (GL7<sup>+</sup>) are red, and the T cell zones (TCR $\beta$ <sup>+</sup>) are blue. Images are at 20x magnification. Quantification of GC size (right). n=4-5 mice/group in 2 pooled independent experiments. ANOVA: \*p<0.05. **B.** TNP-specific IgG affinity collected on day 14 after immunization. n=4 mice/group. Results are representative of 2 independent experiments. **C.** Representative flow cytometry plots (left), percentages (middle), and numbers (right) of FAS<sup>+</sup>GL7<sup>+</sup> GC B cells in the draining LNs. n=7 mice/group. **D.** MFI of surface FAS (left) and GL7 (right) by B220<sup>+</sup>GL7<sup>+</sup> and B220<sup>+</sup>FAS<sup>+</sup> cells, respectively. n=5-6 mice/group. **E.** Percentages of BCL6<sup>+</sup>KI-67<sup>+</sup> B cells in the draining LNs. n=4 mice/group. **F.** MFI of BCL6 (left) and KI-67 (right) in BCL6<sup>+</sup> B cells. n=4 mice/group. **G.** Percentage of FAS<sup>int</sup>GL7<sup>lo</sup>IgD<sup>hi</sup>CCR6<sup>hi</sup> pre-GC B cells (left) and MFI of KI-67 in pre-GC B cells (right). n=7 mice/group from 2 pooled experiments. D-F show a representative experiment out of 3. Data are presented as mean $\pm$ SEM. t-test \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.



**Figure 3. *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice have a normal Tfh percentage and phenotype after immunization with TD antigen.** *Cd4-Cre<sup>Tg</sup>Dock8<sup>flox/flox</sup>* mice and controls were immunized with TNP-KLH in the hock. Draining LNs were analyzed 7 days after immunization. **A.** Representative FACS plots (left), percentages (middle) and numbers of CXCR5<sup>+</sup>PD1<sup>+</sup> Tfh cells (right). Pooled results from 3 individual experiments, n=9 mice/group **B.** Ratio of Tfr:Tfh cells. **C.** MFI of CXCR5, PD1, and ICOS expression by Tfh cells. **D.** Intracellular BCL6 expression by Tfh cells. **E.** MFI of CD150, CD84, and CD40L expression by Tfh cells (left). qPCR analysis of *Sh2d1a* mRNA expression in sorted Tfh cells (right). Results are expressed as fold increase of the *Sh2d1a* mRNA:β2 microglobulin mRNA ratio relative to control. **F.** Intracellular expression of IL-4 and IL-21 by Tfh cells stimulated for 4 hours with phorbol-12,13-dibutyrate and ionomycin. **G.** Percentage of T-B cell conjugates out of total CD4<sup>+</sup> T cells isolated from *Dock8*<sup>-/-</sup> OTII and WT OTII mice incubated for 3 h with LPS stimulated WT B cells pulsed with OVA<sub>323-339</sub>. **H.** Sorted CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup>CD25<sup>-</sup>CD19<sup>-</sup> Tfh cells from the draining LNs were incubated with CD19<sup>+</sup> B cells sorted from the LNs of WT mice in the presence of soluble anti-CD3 and anti-IgM. Cell surface expression of GL7, GLUT1, and IgG1 by B cells after 6 days in culture. Results in B-H are representative of 3 independent experiments. Each data point represents an individual mouse. Data are presented as mean±SEM. t-test \*p<0.05



**Figure 4. DOCK8 deficient CD4<sup>+</sup> T cells have decreased migration into GCs and impaired activation of LFA-1. A,B.** CD4<sup>+</sup>CD45.2<sup>+</sup>OT-II WT or DOCK8-deficient T cells were adoptively transferred into CD45.1<sup>+</sup> WT mice. Recipients were immunized with NP-OVA in the hock, and popliteal LNs were analyzed on day 9 post-immunization. (A) The number of OTII CD45.2<sup>+</sup> T cells found in the draining LNs of recipient mice were analyzed by flow cytometry. (B) The localization within the draining LNs of transferred CD4<sup>+</sup>CD45.2<sup>+</sup>OT-II WT or DOCK8-deficient T cells were examined by immunofluorescence microscopy. Representative photomicrographs at 20x magnification (left). B cell follicles (IgD<sup>+</sup>) are green, GCs (GL7<sup>+</sup>) are red, CD45.2<sup>+</sup> T cells are white, and the T cell zones (CD3<sup>+</sup>) are blue. Percentage of CD45.2<sup>+</sup> in GCs relative to the total CD45.2<sup>+</sup> cells in GC plus the surrounding follicle normalized to the GC and follicle area (right). (n=5 mice/group). **C.** MFI of total surface LFA-1 expression on CD4<sup>+</sup> T cells from *Dock8*<sup>-/-</sup> mice and controls. **D.** Number of adherent CD4<sup>+</sup> T cells, stimulated with anti-CD3 or PMA from *Dock8*<sup>-/-</sup> mice and controls, to ICAM-1, 10 min after application to a flow chamber with a flow rate of 0.75 dynes/cm<sup>2</sup>. Five fields were examined per slide. **E.** Representative histograms (left) and MFI of LFA-1 surface expression (right) by unstimulated and anti-CD3 stimulated CD4<sup>+</sup> T cells from DOCK8 deficient patients and healthy shipping controls (n=3/group). MFI values were normalized to the mean value of the shipping controls. **F.** Representative histograms (left) and MFI of activated LFA-1 expression (right) by unstimulated and anti-CD3 stimulated CD4<sup>+</sup> T cells from DOCK8 deficient patients and healthy shipping controls (n=3/group). MFI values were normalized to the mean value of the unstimulated shipping controls. Results in C and D are of 3 independent experiments each with 3 mice/group. Data in A-F are presented as mean±SEM. In A-C, t-test \*p<0.05. In D-E, one-way ANOVA with Tukey's multiple comparisons \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.