Germline-activating mutations in PIK3CD compromise B cell development and function

Danielle T. Avery1,*1, Alisa Kane1,2,3,4,5, Tina Nguyen1,2, Anthony Lau1,2, Akira Nguyen1,2, Helen Lenthall1, Kathryn Payne1, Wei Shi6,7, Henry Brigden1, Elise French1, Julia Bier1,2, Jana R. Hermes1, David Zahra1, William A. Sewell1,2,3, Danyal Butt1,2,3, Michael Elliott5,10, Kaan Boztug1,11,12,13, Isabelle Meyts1,4, Sharon Choo4,5, Peter Hsu1,2, Melanie Wong5,16, Lucinda J. Berglund5,12,18, Paul Gray1,19, Michael O’Sullivan20, Theresa Cole15, Steven M. Holland21, Cindy S. Ma1,2,5, Christoph Burkhardt22, Lynn M. Corcoran1,2, Tri Giang Phan1,2,5, Robert Brink1,2, Gulbu Uzel21, Elissa K. Deenick1,2,5,*5, and Stuart G. Tangye1,2,5,**

Gain-of-function (GOF) mutations in PIK3CD, encoding the p110δ subunit of phosphatidylinositol 3-kinase (PI3K), cause a primary immunodeficiency. Affected individuals display impaired humoral immune responses following infection or immunization. To establish mechanisms underlying these immune defects, we studied a large cohort of patients with PIK3CD GOF mutations and established a novel mouse model using CRISPR/Cas9-mediated gene editing to introduce a common pathogenic mutation in Pik3cd. In both species, hyperactive PI3K severely affected B cell development and differentiation in the bone marrow and the periphery. Furthermore, PI3K GOF B cells exhibited intrinsic defects in class-switch recombination (CSR) due to impaired induction of activation-induced cytidine deaminase (AID) and failure to acquire a plasmablast gene signature and phenotype. Importantly, defects in CSR, AID expression, and Ig secretion were restored by leniolisib, a specific p110δ inhibitor. Our findings reveal key roles for balanced PI3K signaling in B cell development and long-lived humoral immunity and memory and establish the validity of treating affected individuals with p110δ inhibitors.

Introduction

B cell development occurs in the bone marrow (BM) and involves the progressive maturation of pluripotent hematopoietic stem cells into populations of progenitor (pro-B), precursor (preB), and immature B cells (Uckun, 1990; LeBien and Tedder, 2008). The early stages of B cell development also require the rearrangement of genes encoding the B cell antigen (Ag) receptor (BCR), which is responsible for recognizing specific Ag (Uckun, 1990; LeBien and Tedder, 2008). Immature B cells that express a functional, nonself reactive BCR are then exported to the periphery as transitional B cells, where they undergo final maturation into immunocompetent naive B cells capable of surveying the host for the presence of foreign Ags (Goodnow, 2007).

While B cells play numerous roles in host defense against infection (LeBien and Tedder, 2008), their main function is to produce antibodies (Abs) that neutralize and clear invading pathogens from the host (Goodnow et al., 2010; Tangye et al., 2013, 2015). Following Ag stimulation, naive B cells rapidly become short-lived plasmablasts that localize to extrafollicular regions of lymphoid tissues, or they seed germinal centers (GCs) in lymphoid follicles where, with help from CD4+ T cells, they undergo somatic hypermutation (SHM), affinity maturation,
and differentiation into long-lived memory and plasma cells (PCs; Goodnow et al., 2010; Tangye et al., 2013, 2015). These processes are controlled by signals received through the BCR, CD40, adhesion molecules, and receptors for cytokines and chemokines. A key effector downstream of these receptors is the phosphatidylinositide 3-kinase (PI3K) pathway (Okkenhaug and Vanhaesebroeck, 2003; Okkenhaug, 2013).

There are three classes of PI3K (Class IA, B; II; III) with Class IA PI3K being the predominant type involved in lymphocyte signaling (Okkenhaug and Vanhaesebroeck, 2003; Okkenhaug, 2013). Class IA PI3Ks (hereafter referred to as PI3K) are heterodimers, comprising a regulatory and catalytic subunit. Several isoforms of the regulatory (p85α, p85β, and p55) and catalytic (p110α, β, or δ) subunits have been identified. While p110δ is largely restricted to leukocytes (Okkenhaug and Vanhaesebroeck, 2003; Okkenhaug, 2013), PI3K is activated following engagement of the BCR or CD40, and its activation can be enhanced by coengagement of other receptors, such as CD19, BAFF-R and cyto/chemokine receptors (Ren et al., 1994; Aagaard-Tillery and Jelinek, 1996; Andjelic et al., 2000; Okkenhaug and Vanhaesebroeck, 2003; Okkenhaug, 2013). In B cells, PI3K becomes activated following p85-dependent recruitment to CD19 or the intracellular adaptor BCAP. This interaction represses the inhibitory function of p85, allowing activation of catalytic p110. PI3K converts membrane phosphatidylinositol-(4,5)-bisphosphate (PIP2) to phosphatidylinositol-(3,4,5)-trisphosphate (PIP3), which recruits Tec family kinases (ITK and SHIP, which reduce PIP3 levels by converting it to PIP2 and phosphatidylinositol-(3,4)-bisphosphate (PIP2), which recruits Tec family kinases (ITK and BTK) and the serine/threonine kinase Akt to the inner plasma membrane of the cell. Anchoring of these kinases results in their activation, which then activate intracellular substrates and additional signaling pathways (Ras/MAPK, PKC, NFκB, and Akt/mTOR/FOXO1; Okkenhaug and Vanhaesebroeck, 2003; Okkenhaug, 2013). The action of PI3K is regulated by the lipid phosphatases PTEN (phosphatase and tensin homologue) and SHIP, which reduce PIP3 levels by converting it to PIP2 and phosphatidylinositol-(3,4)-bisphosphate, respectively (Okkenhaug and Vanhaesebroeck, 2003; Okkenhaug, 2013). Thus, production of PIP3 by the balanced functions of PI3K and PTEN initiates activation of the major signaling pathways downstream of Ag, costimulatory and cytokine receptors in B cells that are critical for survival, growth, differentiation, and metabolism.

Analysis of genetically modified mice that lack p85α or p110δ, or express catalytically inactive p110δ, confirmed the importance of PI3K in B cell development and function. Compared with WT mice, these mutant mice have ~50% fewer splenic folliclar B cells, and a severe reduction in marginal zone (MZ) and B1 B cells (Fruman et al., 1999; Suzuki et al., 1999; Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002; Srivivasan et al., 2009). More striking were the dramatically blunted Ab responses and generation of memory cells to T-dependent (TD) Ags, consistent with impaired GC formation in vivo and poor proliferation and survival of activated mutant B cells in vitro. PI3K also inhibits isotype switching by suppressing induction of Aicda (Omori et al., 2006; Dengler et al., 2008), encoding activation-induced cytidine deaminase (AID), or Ig germline transcripts (Dominguez-Sola et al., 2015; Sander et al., 2015), which are required for class switch recombination (CSR). These findings illustrate that PI3K-dependent signaling is required for B cell development, survival and eliciting TD Ab responses. Consistent with its role in restraining PI3K function, PTEN deficiency resulted in increased B cell numbers and serum IgM in vivo, and increased survival, proliferation, and Akt activation in vitro in response to BCR, CD40, or TLR signaling (Anzelon et al., 2003; Suzuki et al., 2003). Paradoxically, PTEN deficiency also resulted in poor GC and TD Ab responses and impaired CSR in vivo (Anzelon et al., 2003; Suzuki et al., 2003; Sander et al., 2015). Interestingly, conditional deletion of PI3K p110δ in CD4+ T cells recapitulated the defect in humoral immune responses in germline-targeted Pik3cd-deficient mice (Rolf et al., 2010), suggesting an additional key B cell extrinsic function for PI3K signaling in regulating TD B cell differentiation. Thus, balanced signaling via PI3K is necessary for qualitatively and quantitatively robust humoral immune responses.

Recently, germline heterozygous gain-of-function (GOF) mutations in Pik3cd, encoding catalytic p110δ, have been identified to cause a primary immunodeficiency (Jou et al., 2006; Angulo et al., 2013; Crank et al., 2014; Kracker et al., 2014; Lucas et al., 2014a; Hartman et al., 2015; Elgizouli et al., 2016; Coulter et al., 2017). Affected individuals present with recurrent sinonasal infections, lymphadenopathy, splenomegaly, and viremia due to uncontrolled infection by human herpes viruses. While these patients have normal/elevated levels of IgM, variable levels of IgG and low/normal levels of IgA, Ag-specific Ab titres against protein- and polysaccharide-containing vaccines are consistently low (Jou et al., 2006; Angulo et al., 2013; Crank et al., 2014; Deau et al., 2014; Kracker et al., 2014; Lucas et al., 2014a; Hartman et al., 2015; Elgizouli et al., 2016; Coulter et al., 2017). Thus, Pik3cd GOF mutations underlie a novel human immunodeficiency disorder thereby highlighting the complex regulation of PI3K signaling. Despite this, the mechanism(s) underlying the cellular defects due to Pik3cd GOF mutations remains unknown. To delineate requirements for p110δ in B cell function, we have now examined B cell development and differentiation in a large cohort of individuals with Pik3cd GOF mutations, as well as a corresponding CRISPR/Cas9 gene-edited mouse model.

Results
Gain of function mutations in Pik3cd impede human B cell development and differentiation in vivo
Ex vivo analysis of B cells from Pik3cd GOF patients revealed elevated levels of phosphorylated ribosomal S6 protein (pS6), which is downstream of mTOR, compared with B cells from healthy donors, confirming hyperactive PI3K signaling (Fig. S1 A). The key clinical features of patients with Pik3cd GOF mutations (recurrent respiratory tract infections, increased serum IgM, concomitant hypogammaglobulinemia, impaired humoral immune responses following infection or vaccination; Angulo et al., 2013; Lucas et al., 2014a; Coulter et al., 2017) point to a defect in B cell development and/or function. To investigate this, we assessed the proportions and phenotype of distinct B cell subsets in a large cohort of affected individuals. Our cohort comprised 39 patients from 27 different families. The mean age of the Pik3cd GOF patients was 18 yr (range: 6–65 yr), and 29/39 (~74%) carried the common E1021K mutation. Analysis of the B cell compartment
revealed comparable frequencies of CD20+ B cells in PIK3CD GOF patients and healthy controls (Fig. 1A; controls: 9.8 ± 0.8%, n = 45; patients: 11.5 ± 1.3%, n = 39; mean ± SEM). However, the proportion of transitional B cells in populations of transitional, naive, and memory B cell subsets (Cuss et al., 2006; Avery et al., 2010; Suryani et al., 2010) revealed marked differences. Specifically, the proportions of transitional B cells were significantly increased (controls: 13.5 ± 1.0%, n = 60; patients: 53.4 ± 3.1%, n = 38), while those of naive B cells (controls: 62.9 ± 1.2%, patients: 38.7 ± 2.8%) and memory B cells (controls: 21.5 ± 1.8%; patients: 6.3 ± 0.7%) were significantly reduced in PIK3CD GOF patients compared with healthy controls (Fig. 1B). To extend the analysis of defects in B cell differentiation, we also determined proportions of class-switched memory B cells. In healthy donors, ~20–25% of memory B cells express IgG or IgA (Avery et al., 2010; Fig. 1C). In contrast, on average <10% of memory B cells in PIK3CD GOF patients expressed IgG or IgA (Fig. 1C). Thus, PIK3CD GOF mutations not only compromised the ability of affected individuals to generate a normal memory B cell pool, but also impaired isotype switching, yielding fewer class switched B cells.

The proportion of transitional B cells in peripheral blood of healthy donors is highest at birth and rapidly declines within the first 5 yr; before continuing to decline at a slower rate over subsequent years (Sims et al., 2005; Cuss et al., 2006; Morbach et al., 2010). Conversely, memory B cells are absent from umbilical cord blood, progressively increase over the first two decades of life, and then plateau after ~25 yr of age (Agematsu et al., 1997; Morbach et al., 2010). As ~70% of our cohort of individuals with PIK3CD GOF mutations were aged between 5 and 20 yr, it was possible that the skewing in composition of the B cell compartment reflected differences in the mean age of the patient cohort compared with that of healthy controls (18.0 vs. 28.5 yr). To investigate this, we determined proportions of B cell subsets according to the age of individual donors and patients. This analysis indeed confirmed the reciprocal relationship between transitional and memory B cells with age in both healthy donors and PIK3CD GOF mutant patients (Fig. 1D). However, it is clear that the average proportion of transitional B cells in the patients exceeded those in controls, while those of memory B cells in healthy donors were greater than in the patients, irrespective of age (Fig. 1D). Similar findings were made when class switching in the memory B cell compartment was analyzed with respect to age (not shown). Thus, PIK3CD GOF mutations profoundly alter B cell development and differentiation.

**PIK3CD GOF mutations result in the accumulation of early transitional B cells in the periphery**

Analysis of the expanded population of transitional B cells in individuals with PIK3CD GOF mutations revealed increased surface expression of CD10 compared with corresponding transitional B cells from healthy donors (see contour plots in Fig. 1B). As CD10 decreases on human progenitor cells as they progress through successive stages of B cell development (Uckun, 1990), we questioned whether transitional B cells in PIK3CD GOF patients were developmentally less mature than those detected in healthy donors. To address this, we determined expression of a suite of molecules that have previously been established to increase or decrease as transitional B cells mature into naive B cells, which then differentiate into memory B cells (Sims et al., 2005; Cuss et al., 2006; Avery et al., 2010; Suryani et al., 2010). Consistent with previous studies, CD5 and CD38 were highly expressed on transitional B cells, and then greatly down-regulated on naive and memory B cells (Sims et al., 2005; Cuss et al., 2006; Suryani et al., 2010). IgM is also reduced as transitional B cells mature into naive B cells (not shown). In contrast, levels of CD44 and Bcl-2 increase as transitional B cells develop into naive B cells and subsequently differentiate into memory B cells. Levels of other surface receptors such as CD21, CD23, CCR7, CXCR4, and CXCR5 also increase as transitional B cells develop into naive B cells, but are then down-regulated during naive→memory differentiation (Fig. 2).

Although the patterns of expression of these molecules by B cell subsets in PIK3CD GOF patients mirrored those of B cells from healthy controls, there were marked differences in the absolute levels of expression. Thus, CD5 and CD38 were approximately threefold higher on transitional and naive B cells from PIK3CD GOF patients than on controls, and remained detectable on PIK3CD GOF memory B cells (Fig. 2, A and B). IgM also remained greatly increased (greater than threefold) on PIK3CD GOF transitional and naive B cells (not shown). CD21, CD44, Bcl-2, CCR7, CXCR4, and CXCR5 were up to threefold lower on B cell subsets from PIK3CD GOF patients compared with controls (Fig. 2, C, E, G–I). CD23 was also reduced on PIK3CD GOF transitional and naive B cells, and failed to be down-regulated on memory B cells (Fig. 2D). Since PI3K is activated downstream of CD19, and CD81 forms a complex with CD19 and other B cell coreceptors (Carter and Barrington, 2004), we also assessed expression of these molecules. CD19 was significantly reduced on naive B cells, while CD81 was significantly increased on transitional B cells, from PIK3CD GOF patients compared with controls (Fig. 2F; not shown).

There are at least two subsets of transitional B cells detectable in human peripheral blood (Anolik et al., 2007; Palanichamy et al., 2009; Suryani et al., 2010). Our previous studies delineated CD21hi and CD21lo subsets of transitional B cells, with the CD21hi subset being less mature and a precursor of the CD21lo subset (Suryani et al., 2010). The above phenotyping data revealed that the majority of transitional B cells in PIK3CD GOF patients corresponded to the CD21lo subset. They also suggested that CD20+CD10+CD27+ B cells in PIK3CD GOF patients were less mature than corresponding naive B cells from healthy donors, since the former exhibited a phenotype more similar to that of normal transitional rather than naive B cells. To further investigate these B cell defects, we determined expression levels of genes differentially expressed during peripheral B cell development. LEFI and DTX1 are more highly expressed in CD21hi than in CD21lo transitional B cells (Suryani et al., 2010). Quantitative PCR (qPCR) analysis revealed DTX1 was expressed at 5- and 10-fold–higher levels in transitional and naive B cells, respectively, from PIK3CD GOF patients than in these cells from health donors (Fig. 1B). Similarly, while LEFI is expressed in transitional B cells and extinguished in naive B cells from healthy donors, it continued to be expressed in PIK3CD GOF naive B cells (Fig. 1B). Thus, the circulating B cell compartment of patients with PIK3CD GOF mutations is enriched for cells at early stages of B cell development.
Figure 1. **GOF mutations in PIK3CD arrest peripheral B cell development and differentiation.** PBMCs from healthy donors (n = 45–60) and patients with PIK3CD GOF mutations (n = 21–39) were labeled with mAbs against CD20, CD10, CD27, IgG, or IgA. The proportions of (A) B (CD20⁺) cells within the lymphocyte gate, (B) transitional, naive, and memory cells within the B cell population, and (C) IgG⁺ and IgA⁺ cells within the memory population were determined by flow cytometry. Histogram and contour plots are representative of healthy donors or PIK3CD GOF patients. Each symbol in the summary graphs corresponds to an individual donor or patient; horizontal bars represent the mean. Significant differences were determined by unpaired Student’s t tests. ****, P < 0.0001. (D) Proportions of transitional (left panel) and memory (right panel) B cells in healthy donors (black) and PIK3CD GOF patients (red) were determined as a function of age.
Figure 2. **Peripheral B cells in PIK3CD GOF patients have an immature phenotype.** Expression of (A) CD5, (B) CD38, (C) CD21, (D) CD23, (E) CD44, (F) CD19, (G) CCR7, (H) CXCR4, (I) CXCR5, or (J) BCL2 was determined on transitional, naive, and memory B cells in peripheral blood of healthy donors or patients with PIK3CD GOF mutations (n = 6–30). Histogram plots are representative of healthy donors (upper panels) or PIK3CD GOF patients (lower panels). The bar graphs depict the geometric mean fluorescence intensity (± SEM) of each indicated molecule. Significant differences were determined by unpaired Student’s t tests. *, P < 0.05; **, P < 0.01; ****, P < 0.0001.
**PIK3CD GOF mutations cause aberrant B cell development in the bone marrow**

B cell development occurs in the BM, where hematopoietic stem cells undergo progressive steps to yield pro-B, pre-B, and immature B cells before being exported to the periphery as transitional cells (Uckun, 1990). To further explore the nature of the defect in B cell development observed in the peripheral blood, we quantified the proportions of pro-B (CD19^+^CD34^+^CD10^-^CD20^-^IgM^-_), pre-B (CD19^+^CD34^-^CD10^-^CD20^-^IgM^-_), pre-BI (CD19^-^CD34^-^CD10^-^CD20^-^IgM^-_), immature (CD19^-^CD34^-^CD10^-^CD20^-^IgM^-_), and recirculating mature (CD19^-^CD34^-^CD10^-^CD20^-^IgM^-_) B cells in the BM of PIK3CD GOF patients. Proportions of pro-B and pre-BI cells in controls and patients were similar (Fig. 3, A, B, and D). However, compared with BM from healthy donors, we found significantly increased proportions of pre-BII and immature B cells, and marked reductions in mature recirculating B cells (CD19^-^CD20^-^CD10^-_) in BM from PIK3CD GOF patients (Fig. 3, A, B, and D).

To extend the findings from analysis of peripheral blood, we enumerated naive and memory B cells in the BM. When CD19^+^ B cells were stained with mAbs against CD34, CD19, CD20, and CD27, proportions of pro-B, pre-BI, pre-BII, immature, and mature (CD19^-^CD34^-^CD10^-^CD20^-^IgM^-_) B cells were determined. (A–C) Contour plots in show CD34 versus CD10 staining to identify pro-B cells, which were then further analyzed for pre-BI, pre-BII, immature, and recirculating mature according to differential expression of CD20 and CD10. (D) Mean ± SEM of the different subsets of B cells in the BM. (E) Representative contour plots showing CD10 versus CD27 staining on CD20^+^ B cells, and (F) mean ± SEM of CD10^-^CD27^-, naive (CD20^-^CD10^-^CD27^+) and memory (CD20^-^CD27^+) B cells in the BM. Significant differences were determined by Student’s t test. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
PIK3CD mutations selectively compromize the differentiation of B cells into class switched Ig secreting plasmablasts

Impaired humoral immune responses following natural infection or immunization characterized by reduced levels of total and Ag-specific serum Ig are hallmarks of the immunodeficiency associated with PIK3CD GOF mutations (Angulo et al., 2013; Lucas et al., 2014a; Coulter et al., 2017). Data from mice indicated that germline targeting of Pik3cd, or conditional loss of Pten in B cells (Fruman et al., 1999; Suzuki et al., 1999; Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002; Suzuki et al., 2003; Omori et al., 2006). Thus, regulated function of PI3K signaling is critical for generating robust and long-lived Ab responses.

To determine the impact of PIK3CD GOF mutations on the function of human B cells, transitional and naive B cells were sorted from the peripheral blood of healthy donors and patients and subjected to in vitro culture. Proliferation of naive PIK3CD GOF B cells, as determined by dilution of CFSE, in response to mimics of TD (e.g., CD40L ± cytokines) and TI (e.g., BCR and TLR engagement) stimulation was comparable to, or slightly greater than, that of naive B cells from healthy controls (Fig. 5C). Thus, the paucity of class switched memory B cells and impaired humoral immune responses in PIK3CD GOF patients cannot be explained by a proliferation defect in naive B cells.

Next, we assessed differentiation of naive B cells into plasmablasts upon stimulation with CD40L and IL-21 for 5 d. This combination of stimuli is not only a potent means of inducing human B cell proliferation and differentiation (Moenes and Tangye, 2014), but both CD40L (Ren et al., 1994; Aagaard-Tillery and Jelinek, 1996; Andjelic et al., 2000) and IL-21 (Ostiguy et al., 2007; Zeng et al., 2007) individually activate PI3K. Indeed, CD40-induced proliferation and IL-21–induced up-regulation of CD86 are reduced or abolished in murine Pik3cd and Pik3r1 mutant B cells (Fruman et al., 1999; Suzuki et al., 1999; Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002; Attridge et al., 2014). Under these culture conditions, comparable proportions (~3–8%) of naive B cells from healthy controls or patients up-regulated expression of CD38 and CD27 and down-regulated CD20 (CD38hi CD27lo CD20lo), thereby acquiring a plasmablast phenotype (Avery et al., 2005; Fig. 4, A and B). Consistent with this, induction of PRDM1 mRNA (encoding Blimp-1; Fig. 4C) as well as of Blimp-1 protein in plasmablasts (Fig. 4D) was intact for patients’ naive B cells. As AID contributes to the B cell response by inducing CSR (Durandy et al., 2007), we determined induction of AICDA mRNA in activated naive B cells. While AICDA was induced in PIK3CD GOF naive B cells by CD40L/IL-21, it tended to be less than levels observed in naive B cells from healthy donors (Fig. 4E).

To explore B cell differentiation in vitro in more detail, we determined the quality of the plasmablast response. Intracellular staining revealed that ~60% of plasmablasts generated from normal naive B cells expressed IgG, with the remaining expressing IgM or IgA (~20% of each; Fig. 4, F and G). Strikingly, the majority of plasmablasts generated from PIK3CD GOF naive B cells continued to express IgM, and only ~10% had undergone CSR to IgG (Fig. 4, F and G). Remarkably, class switching by PIK3CD GOF naive B cells to IgA was intact (Fig. 4, F and G). Analysis of Ig secretion by in vitro stimulated naive B cells confirmed these findings, with a sharp reduction (~10-fold) in levels of IgG, but normal levels of IgM and IgA (Fig. 4H). The poor secretion of IgG by PIK3CD GOF naive B cells did not reflect delayed kinetics of differentiation into IgG-secreting cells, because this defect was also noted after 7 d of culture (Fig. 4H).

We also tested the functionality of transitional cells, as these cells dominate the B cell population in PIK3CD GOF patients (Fig. 1B). Similar to naive cells, induction of PRDM1 mRNA (Fig. 4C) and production of IgM (Fig. 4I) by PIK3CD GOF transitional B cells in response to CD40L/IL-21 stimulation was intact. AICDA expression was also induced in activated PIK3CD GOF transitional B cells and modestly reduced compared with control transitional B cells (Fig. 4E). Despite these findings, PIK3CD GOF transitional B cells were unable to produce normal amounts of IgG (~50-fold reduction compared with controls after 5 and 7 d of in vitro culture; Fig. 4I). Notably, PIK3CD GOF transitional B cells also exhibited a significant defect in switching to IgA at both time points examined (Fig. 4I). Collectively, these findings establish that while hyperactive PI3K signaling has no effect on proliferation of human B cells, nor their initial differentiation into IgG-secreting cells, it impairs the ability of naive B cells to undergo class switching to IgG and of transitional B cells to produce IgG and IgA.

A gene set associated with plasmablast differentiation is poorly induced in PIK3CD GOF transitional B cells

To explore molecular defects underlying impaired differentiation of PIK3CD GOF B cells, we determined gene expression profiles of transitional B cell subsets from healthy donors and PIK3CD GOF patients following in vitro culture with CD40L alone or together with IL-21. Modulation of key genes involved in IL-21–mediated differentiation of human B cells into plasmablasts was largely unaffected by PIK3CD GOF mutations. Specifically, PRDM1, BXPI, IRF4, MZBI, and ILL0 were induced, while CCR7 and BACH2 were down-regulated in control and PIK3CD GOF transitional B cells following culture with CD40L/IL-21 compared with cells cultured with CD40L alone (Fig. 5A). In contrast, PAX5 was down-regulated in control, but less so in PIK3CD GOF transitional B cells (Fig. 5A; confirmed by qPCR, not shown). Unbiased analysis and comparison of transcriptomes from CD40L/IL-21–stimu-
lated control and PIK3CD GOF transitional B cells identified 346 differentially expressed genes (DEGs; absolute fold change >1.5, false discovery rate [FDR] <0.05; Fig. S2 A). Non-negative matrix factorization (NMF; Brunet et al., 2004; Kim and Park, 2007) was performed on this set of DEGs to identify the key molecular drivers underlying differences in the gene expression profiles of...
normal and PIK3CD GOF activated transitional B cells (Fig. 5 B). Among the driver genes highly expressed by activated PIK3CD GOF relative to normal transitional B cells were SIGLEC6, which is expressed on human B cells and inhibits their proliferation and differentiation (Patel et al., 1999; Kardava et al., 2011). MPEGI, KMO, the CD20-like gene MS4A7 (Fig. 5 B), as well as MS4A4A1 (CD20), CD72, CD22, CD84, FcgR2C, and CD24 (Fig. S2 A), which are all typically down-regulated as B cells differentiate into PCs (Ellyard et al., 2004; Avery et al., 2005; Jourdan et al., 2011). Additionally, CD27 and SLAMF7, which are highly expressed by primary human PCs (Jung et al., 2000; Ellyard et al., 2004; Good et al., 2009), were up-regulated in normal, but not PIK3CD GOF, transitional B cells following in vitro stimulation with CD40L/ IL-21 compared with culture with CD40L alone (Fig. 5 B).

We adopted several approaches to confirm these findings relating to differential gene expression in activated control and PIK3CD GOF transitional B cells. First, to ascertain the biological and physiological relevance of the pattern of differentially expressed genes between PIK3CD GOF and control transitional B cells, we performed RNA-seq on B cell subsets isolated from human tonsils. Analysis of this dataset clearly revealed that the genes identified from the microarrays to be highly expressed in activated PIK3CD GOF transitional B cells, but down-regulated in control transitional B cells, are characteristic of resting naive and memory tonsil B cells (i.e., KMO, MPEGI, MS4A7, PTPRJ, RAP GAP12, and SIGLEC6; Fig. S2 B). In contrast, genes up-regulated in normal, but not PIK3CD GOF, transitional B cells following in vitro culture with CD40L/IL-21 were expressed at the highest levels in primary human PCs (i.e., CD27, SLAMF7, ZNF251, SLC29A1, MYO1D, and BEX5; Fig. S2 C; Jung et al., 2000; Ellyard et al., 2004; Good et al., 2009). The RNA-Seq data were next confirmed by qPCR analysis of gene expression in tonsil naive, memory, GC B cells, and PCs isolated from a second cohort of individuals undergoing tonsillectomy. This established that KMO, MS4A7, PTPRJ, RAP GAPA2, and SIGLEC6 are more highly expressed in naive and/or memory B cells, while SLAMF7, ZNF215, GPR15, MYO1D, and BEX5 are highly expressed in PCs (Fig. 5 C and D). Flow cytometric analysis of tonsil B cell subsets confirmed increased expression of Siglec-6 on memory B cells, and reduced expression on PCs (Fig. S2 D), but increased expression of CRACC (SLAMMP7) and CD27 on PCs compared with naive, memory and GC B cells (Fig. S2 E and F). Consistent with this, Siglec-6 was expressed at higher levels in in vitro–activated B cells compared with plasmablasts following culture with CD40L/IL-21, while CRACC and CD27 were induced on plasmablasts generated in vitro from naive B cells in the same cultures (Fig. S2 G). Third, gene set enrichment analysis indicated that activated PIK3CD GOF transitional B cells exhibit gene expression profiles that are more consistent with naive or memory B cells rather than PCs, which, akin to the phenotype of cultured PIK3CD GOF transitional B cells, contrasts with activated transitional B cells from healthy controls (Fig. S2 H). Fourth, we directly measured the ability of PIK3CD GOF transitional B cells to differentiate into plasmablasts in vitro. Strikingly, these B cells failed to give rise to CD38hiCD22hi plasmablasts following culture with CD40L/IL-21 (Fig. 5 E). This starkly contrasted the response of transitional B cells from healthy donors (Fig. 5 E), as well as naive B cells from healthy donors and PIK3CD GOF patients (Fig. 4, A and B). To extend these observations, and validate the differential expression of several of the genes identified by microarray, qPCR was performed using cDNA from activated control and PIK3CD GOF transitional B cells. This revealed markedly increased expression of KMO, MS4A7, PTPRJ, and SIGLEC6, but decreased expression of SLAMF7 and MYO1D by in vitro CD40L/IL-21–activated PIK3CD GOF transitional B cells compared with controls (Fig. 5 F). These data underscore the findings from the microarray, phenotypic, and functional analysis of PIK3CD GOF B cells and collectively establish that induction of a plasmablast transcriptional signature is compromised in PIK3CD GOF transitional B cells, even though these cells can exhibit some features of early differentiation to the Ig-secreting cell lineage.

Development of a mouse model of Pik3cd GOF
To further explore how PI3K overactivation alters B cell development and function we used CRISPR/Cas9 gene editing to introduce a heterozygous E1020K mutation in Pik3cd in the germline of C57BL/6 mice. This is the orthologue of the most common mutation found in the human PIK3CD gene (E1021K). We assessed PI3K activity in these animals by staining splenocytes for phosphorylated Akt (T308 and S473). B cells in these mice displayed significant increases in basal levels of phosphorylation of both T308 and S473 (Fig. 6 A and Fig. S3 A). pS6 protein was similarly increased in B cells of Pik3cdE1020K GOF mice compared with WT mice (Fig. 6 A and Fig. S3 A), consistent with our observations of PIK3CD GOF human B cells (Fig. S1 A). Mice homozygous for the Pik3cdE1020K GOF mutation showed even greater activation of these signaling pathways (Fig. 6 A), indicating a dose-dependent effect of the activating mutation. Importantly, similar to human lymphocytes (Rao et al., 2017), increased levels of pAkt and pS6 in Pik3cdE1020K GOF murine B cells could be reduced by preincubation with leniolisib, a p110δ-specific inhibitor (Fig. S3, A and B; Hoegenauer et al., 2017) demonstrating that heightened activation of these signaling components was PI3K dependent.

Pik3cdE1020K GOF mice allowed us to investigate the effect of overactive PI3K on B cell development in multiple lymphoid organs without any complications of ongoing infections or prior/ current treatments. Analysis of BM revealed an increase in pro-B cells and a decrease in mature recirculating IgD+ cells in Pik3cdE1020K GOF mice compared with WT controls (Fig. 6 B). Analysis of mixed BM chimeras demonstrated that these effects on B cell development in the BM were cell intrinsic (not shown).

Given the decrease in mature B cells in the BM we also examined B cells in the periphery. Spleens of Pik3cdE1020K GOF mice had increased cellularity over WT spleens, which was at least in part due to an increase in total numbers of B cells (data not shown). Staining with CD93 allowed us to identify transitional B cells. This revealed an overall increase in absolute number of transitional B cells (data not shown), which was due to an almost threefold increase in the number of T1 B cells (Fig. 6 C). Interestingly we observed a significant down-regulation of IgM on T1 B cells from Pik3cdE1020K GOF mice compared with those from WT controls (Fig. 6 C). We also noted an increased number of cells that down-regulated IgM within the CD23+ T2/T3 gate, consistent with increased numbers of anergic T3 cells (Fig. 6 C). The
mature (CD93+) B cell pool in Pik3cdE1020K GOF mice was also expanded, with a striking increase in MZ cells. In contrast follicular B cells were only slightly increased (Fig. 6D). We also observed an increase in B1 cells in the spleen, particularly B1a cells (Fig. 6E). Mixed BM chimeras established that these changes in B cell maturation within the spleen were cell intrinsic (not shown). Analysis of B cell populations in the circulation revealed comparable proportions of total B cells in Pik3cdE1020K GOF mice, but increased proportions of B1 and transitional B cells and decreased proportions of mature B cells compared with WT mice (Fig. S3, C–F). This is consistent with our observations of splenomegaly from Pik3cdE1020K GOF mice (Fig. 6) and the blood of PIK3CD GOF patients (Figs. 1 and 2).

**Pik3cd GOF B cells have impaired Ig class switching in vitro**

Analysis of human B cells showed decreased class switched memory cells in vivo and decreased production of switched plasmablasts and secretion of IgG in vitro. To determine whether PI3K overactivation lead to a decrease in switching to distinct IgG subclasses in response to different stimuli, we sorted follicular B cells from the spleens of WT or Pik3cdE1020K GOF mice and stimulated them with anti-CD40 + IL-4, LPS, or LPS + TGF-β to induce CSR to IgG1, IgG2b, or IgG3, respectively (Hodgkin et al., 1996; Hasbold et al., 1998, 2004; Deenick et al., 1999). After 4 d, 50% fewer Pik3cdE1020K GOF follicular B cells were positive for these switched isotypes following culture under the three different conditions compared with B cells from WT mice (Fig. 7, A–C). This was not due to switching to alternative isotypes, as we saw no increases in expression of other IgG subclasses or IgA (data not shown).

As class switching is division linked (Hodgkin et al., 1996; Hasbold et al., 1998, 2004; Deenick et al., 1999), we also labeled B cells with CFSE to determine the percentage of switched cells in each division. The proportion of WT B cells undergoing CSR sharply increased after three divisions, and this continued to increase with subsequent divisions (Fig. 7, A–C), consistent with previous findings (Hodgkin et al., 1996; Hasbold et al., 1998, 2004; Deenick et al., 1999). Pik3cdE1020K GOF B cells also underwent CSR in a division-linked manner, however the percentage of switched cells in each division remained approximately half of that seen for WT cells (Fig. 7, A–C). Thus, similar to our observations for human B cells, activating mutations in PI3K p110δ impede Ig class switching, but not proliferation, by murine B cells.

**Analysis of Pik3cd GOF B cell responses in vivo revealed decreased Ig class switching but normal expansion and affinity maturation**

A benefit of a mouse model is that it allows us to track the activation and differentiation of B cells in vivo. Thus, we crossed the Pik3cdE1020K GOF mice to transgenic mice expressing a BCR recognizing hen egg lysozyme (HEL), hereafter referred to as SwHEL mice (Brink et al., 2015). To determine the intrinsic effect of overactive PI3K on B cell responses, we transferred WT or Pik3cdE1020K GOF SWHEL cells to congenic WT recipients which were then immunized with SRBC conjugated to a lower affinity variant of HEL (HEL-2x) (Brink et al., 2015). When tracked over a 10-d period, expansion of the Pik3cdE1020K GOF B cells was comparable to WT B cells (Fig. 8A). Similarly we saw no difference in the percentages of SWHEL B cells that became plasmablasts or GC cells (Fig. 8B), suggesting PI3K activity does not control the decision between plasmablast and GC fates. In this SWHEL/SRBC system a large proportion of responding B cells switch to IgG1 (Brink et al., 2015). Analysis of B cell subsets revealed that ~50% fewer Pik3cdE1020K GOF plasmablasts and GC B cells had switched to IgG1 compared with WT B cells (Fig. 8C). Again, this was not due to an increase in switching to alternative isotypes, as ~2-fold more Pik3cdE1020K GOF B cells continued to express IgM (Fig. 8C). To determine how this affected production of Ag-specific antibodies we measured serum levels of anti-HEL antibody of various isotypes 5 d after immunization. Mice that received Pik3cdE1020K GOF SWHEL B cells had a fivefold increase in the levels of anti-HEL IgG antibody (Fig. 8D, B), consistent with the hyper-IgM phenotype often observed in PIK3CD GOF patients (Coulter et al., 2017). Despite the decreased percentage of switched IgG1+ cells we saw no significant decrease in serum IgG1 in Pik3cdE1020K GOF compared with WT mice (Fig. 8D). We also measured levels of serum IgG2a, IgG2b, and IgG3. There were trends for increased IgG3 and decreased IgG2b, but no significant change in IgG2a (Fig. 8D, B; not shown). Similar results were also seen 10 d after immunization (not shown).

An important function of the GC is affinity maturation of the Ab response. To determine whether Pik3cdE1020K GOF disrupts this process within the GC, we sorted IgG1+ SWHEL B cells from mice 10 d after immunization and sequenced the BCR to identify somatic mutations. The average number of mutations per sequence was similar between WT and Pik3cd GOF IgG1+ SWHEL B cells (2.6 vs. 2.5, n = 84 or 91), indicating that PI3K GOF did not affect the overall rate of SHM. Affinity maturation in...
Figure 6. **Mice with overactive PI3K show aberrant B cell development.** (A) B cells were stained intracellularly for phosphorylated Akt (T308 and S473) and S6 (S235/236). Histograms show representative staining from WT (black), Pik3cd<sup>E1020K</sup> heterozygous (Pik3cd<sup>GOF</sup>, red), or Pik3cd<sup>E1020K</sup> homozygous (Pik3cd<sup>homGOF</sup> blue) mice and graphs give MFI relative to WT controls (mean ± SEM, n = 5). (B–D) BM and spleens from WT and Pik3cd<sup>E1020K</sup> heterozygous GOF mice aged 8–12 wk were stained to identify different B cell populations. (B) B cell development in the BM. Flow cytometry plots showing representative staining of IgD versus IgM on B220<sup>+</sup> cells. Numbers are percent IgM<sup>-</sup>IgD<sup>-</sup>, IgM<sup>hi</sup>, and IgD<sup>hi</sup> cells. IgM<sup>-</sup>IgD<sup>-</sup> cells were further gated on CD24 and CD43 to identify pre-pro-, pro- and pre-B cells. Graphs give mean ± SEM (n = 9–12). (C) Transitional cells in the spleen. Flow plots show IgM versus CD23 on B220<sup>+</sup>CD93<sup>+</sup> cells. Graphs show absolute numbers of T1 and T2/T3 cells as well as the percentage of each population that is IgM<sup>hi</sup> (mean ± SEM, n = 9–12). (D) Percentages of follicular (CD21<sup>+</sup>CD23<sup>-</sup>) and MZ (CD21<sup>+</sup>CD23<sup>+</sup>) B cells were determined in the mature B cell population (CD93<sup>-</sup>) of the spleen. Flow plots show representative staining of CD21 versus CD23. Graphs show absolute numbers of follicular and MZ cells (mean ± SEM, n = 10–13). (E) Percentages of B1a (CD19<sup>+</sup>BDCA2<sup>+</sup>B220<sup>+</sup>) or B1b (CD19<sup>+</sup>BDCA2<sup>+</sup>B220<sup>-</sup>) cells were determined. Flow plots show representative staining of B220 versus CD19 gated on CD19<sup>+</sup> cells (upper panel) and CD19 versus CD5 gated on CD19<sup>+</sup>B220<sup>+</sup> cells (lower panel). Graphs show absolute numbers of B1a and B1b cells in the spleen (mean ± SEM, n = 6–8). Significant differences were determined by unpaired Student’s t tests. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
SWHEL B cells responding to HEL-2x is associated with a Y53D mutation, which greatly increases affinity of the receptor for the mutant HEL (Brink et al., 2015). By day 10, >50% of WT cells acquired the Y53D mutation (Fig. 8 F). A similar percentage of Pik3cdE1020K GOF B cells were also found to have the Y53D mutation, indicating that increased PI3K activity did not affect affinity maturation.

Thus despite the many reported roles of PI3K in controlling B cell function, as revealed by germline modification of genes encoding p110δ, p85, or PTEN (Fruman et al., 1999; Suzuki et al., 1999, 2003; Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002; Anzelon et al., 2003; Omori et al., 2006; Dengler et al., 2008; Srinivasan et al., 2009), we found that during an in vivo TD response the primary defect was in switching to IgG. In contrast, other functions such as expansion, GC formation and affinity maturation remained intact.

Inhibition of hyperactive PI3K signaling partially overcomes defects in B cell differentiation

Inhibitors of p110δ have been developed and used to treat several human B cell malignancies (Hewett et al., 2016; Fruman et al., 2017). Leniolisib is a recently developed specific inhibitor of p110δ (Hoegenauer et al., 2017) and has shown safety and clinical efficacy in treating six patients with PIK3CD GOF mutations, including partial corrections of B cell subsets and serum IgM levels (Rao et al., 2017). However it is unknown whether the favorable clinical outcome on humoral immunity results from a direct effect on B cells or is secondary to effects on other immune cell types, such as CD4+ T cells (Rolf et al., 2010). To explore this in more detail, we determined whether leniolisib could improve the function of human and murine B cells with hyperactive PI3K signaling. We examined Ig secretion and class switching by B cells following in vitro stimulation, as defects in these processes were the most robust phenotype observed (Figs. 4 and 7). The concentrations of leniolisib tested were optimized to have a minimal effect on B cell proliferation and survival (not shown). When transitional B cells from healthy donors were cultured with CD40L/IL-21, leniolisib resulted in a concentration-dependent decrease in secretion of IgM and IgG, but had no effect on IgA (Fig. 9, A–C). In contrast, leniolisib had no effect on IgM secretion by PIK3CD GOF transitional B cells, but resulted in significant increases in secretion of the class switched isotypes IgG and IgA (Fig. 9, A–C).

To more directly assess CSR, we cultured murine B cells with anti-CD40/IL-4 and various doses of leniolisib. Leniolisib increased the percentage of IgG1+ cells generated from both WT and Pik3cdE1020K GOF B cells (Fig. 9 D). Importantly, the class switch defect in Pik3cdE1020K GOF murine B cells was completely restored by inhibiting p110δ (Fig. 9 D). Division analysis revealed that this was due to a dramatic increase in switching in the earlier divisions such that, at the higher dose of leniolisib, >20% of both WT and Pik3cdE1020K GOF B cells that had undergone one division had switched to IgG1 compared with <2% in control cultures (Fig. 9 E). Interestingly, while similar switching was observed between WT and GOF cells at the higher dose of leniolisib, at the lower dose there remained a difference between WT and Pik3cdE1020K GOF B cells, indicating a dose-dependent relationship between PI3K activation and switching.

To gain insight into the molecular mechanism for reduced CSR due to PI3K p110δ GOF, we performed qPCR on WT and Pik3cdE1020K GOF B cells to establish expression levels of genes involved in regulating B cell differentiation. Bcl6 and Prdm1 were expressed at normal levels in activated Pik3cdE1020K GOF B cells (not shown). However, induction of Aicda was significantly impaired in Pik3cdE1020K GOF murine B cells following stimulation with anti-CD40L/IL-4 (Fig. 9 F). Strikingly, treatment of Pik3cdE1020K GOF B cells with leniolisib restored Aicda expression to levels observed in WT B cells (Fig. 9 F). Notably, Aicda was also increased in leniolisib-treated WT B cells, consistent with increased class switching to IgG1 by these B cells in the presence of increasing doses of this p110δ inhibitor (Fig. 9 D, E). Indeed, plotting Aicda expression versus IgG1+ B cell defects due to hyperactive PI3K kinase
Figure 8. *Pik3cd GOF* B cells show defective switching but normal expansion and affinity maturation in vivo. WT or *Pik3cd GOF* SWHEL cells were transferred to WT congenic hosts, which were then immunized with HEL-2x-SRBC. (A) The expansion of SW_{HEL} cells was tracked over time (mean ± SEM, n = 3–4 mice per group, representative experiment shown). (B) Percentage of cells with a plasmablast or GC phenotype was determined. (C) Percentage of cells that switched to IgG1 or were unswitched (IgM+) was determined in the plasmablast and GC populations. (D) Levels of HEL-specific serum Ig of various classes at day 5.5 were determined by ELISA (B–D, each linked point shows mean ± SEM [n = 3–5] of single experiment). (E) Donor GC IgG1+ cells were sorted on day 10 and sequenced to identify mutations. Significant differences were determined by paired t tests. **, P < 0.01.
cells for WT and Pik3cdE1020K GOF B cells in the absence and presence of leniolisib revealed a very strong positive correlation (Fig. 9 G). Together, these data reveal that activating mutations in PI3K p110δ impede Ig CSR by reducing AID expression in B cells. Furthermore, pharmacological targeting of the PI3K pathway with leniolisib corrects defects in B cell differentiation by relieving the inhibitory effect of hyperactive PI3K signaling on AID expression.
Discussion

Signaling via PI3K plays a fundamental role in immune function, immune regulation, and humoral immunity. This is evidenced from phenotypes arising in mice with germline deletion or inactivation of the p110δ or p85 subunits of PI3K (Fruman et al., 1999; Suzuki et al., 1999; Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002; Srinivasan et al., 2009), or of PTEN (Anzelon et al., 2003; Suzuki et al., 2003; Sander et al., 2015) which antagonizes PI3K signaling. More strikingly, homozygous mutations in PIK3RI prevented B cell development in humans (Conley et al., 2012; Tang et al., 2018), while heterozygous germline mutations in PIK3CD, PIK3RI, or PTEN result in immune dysregulation evidenced by immunodeficiency, autoimmunity, and B cell malignancy (Jou et al., 2006; Angulo et al., 2013; Crank et al., 2014; Deau et al., 2014; Kracker et al., 2014; Lucas et al., 2014a,b; Hartman et al., 2015; Driessen et al., 2016; Elgizouli et al., 2016; Elkaim et al., 2016; Tsujita et al., 2016; Coulter et al., 2017). These observations from mice and humans underscore the critical requirement for regulated PI3K signaling for immune homeostasis and function. However, mechanisms underlying disease pathogenesis in humans remain incompletely determined.

By analyzing a large cohort of patients (n = 39) with activating mutations in PIK3CD and developing a novel mouse model of Pik3cc^{E1020K} GOF by CRISPR/Cas9-mediated genome editing, we have now revealed key functions for PI3K in B cell development and differentiation. B cell development in human BM was perturbed at the pre-BII and immature stages, with an aberrant accumulation of these cells and corresponding reductions in recirculating mature B cells. This extends a previous study that confirmed B cell development in PIK3CD GOF revealed that heterozygous PIK3CD p110δ GOF mice also had increased numbers of B1 and MZ B cells, akin to B cell specific PTEN-deficient mice (Anzelon et al., 2003; Suzuki et al., 2003). Thus, mutations that result in PI3K p110δ GOF strongly compromise successful B cell development in the BM and periphery leading to a skewed B cell compartment.

PI3K p110δ GOF also resulted in several functional B cell defects. Memory B cells were reduced in PIK3CD GOF patients. Among the contracted population of memory B cells, the proportions that had undergone Ig isotype switching were significantly reduced. By studying our Pik3cc^{E1020K} GOF mouse model we confirmed that this in vivo defect in switching was B cell intrinsic and affected all IgG subclasses. Activated PI3K p110δ GOF B cells from humans and mice exhibited defective class switching and secretion of IgG and IgA. Murine PI3K p110δ GOF B cells expressed lower levels of Aicda mRNA than WT B cells. Importantly, the p110δ inhibitor leniolisib improved secretion of IgG and IgA by PIK3CD GOF human B cells, and completely restored not only CSR but also Aicda expression in Pik3cc^{E1020K} GOF murine B cells. Indeed, manipulating PI3K function either by introduction of the GOF allele or inhibition with leniolisib revealed a strong correlation between PI3K activity, AID expression, and IgG class switching. Thus, the molecular mechanism underlying impaired CSR due to PI3K GOF most likely results from reduced expression of AID. This conclusion concurs with previous studies suggesting PI3K regulates CSR in murine B cells by repressing AID (Omori et al., 2006; Dengler et al., 2008; Compagno et al., 2017). Further analysis of the microarray data using the REACTOME database revealed significantly greater expression of components of pathways involved in DNA damage responses in activated transitional B cells from healthy donors than from PIK3CD GOF patients (Table S1). The DNA damage response is required to maintain genomic stability and is induced in response to the AID-mediated DNA breaks that are necessary for CSR (Daniel and Nussenzweig, 2013). This is further evidence of reduced AID function due to hyperactive PI3K p110δ, which underlies impaired CSR in these B cells. Alternatively, decreased expression of molecules involved in the DNA damage response and AID-mediated mismatch repair, such as EXO1 (Table S1; Eccleston et al., 2011) may themselves compromise CSR. It is also possible that B cell extrinsic defects arising from PI3K p110δ GOF, such as compromised function of CD4+ T cells, especially Tfh cells (Rolf et al., 2010), compound these B cell intrinsic defects in effector function. This is currently being investigated.

In contrast to decreased CSR, SHM (which is also dependent on AID; Durandy et al., 2007) was intact in murine PI3K p110δ B cells, suggesting that the decrease in AID expression detected was insufficient to ablate the function of AID in SHM. This is consistent with impaired class switching (Fig. 1), but intact SHM (Angulo et al., 2013), in memory B cells from PIK3CD GOF individuals, and also the discovery of some patients with biallelic mutations in the C-terminal domain of AICDA whose B cells are unable to undergo CSR but exhibit normal levels of SHM (Durandy et al., 2007).

Although PIK3CD GOF B cells exhibited hallmarks of Ig-secreting cell differentiation in vitro (IgM secretion and BLIMP-1 expression), the magnitude and quality of this process was compromised, particularly in transitional B cells. These differences in Ig secretion by transitional B cells from controls and PIK3CD GOF patients may reflect differences in the composition of the transitional B cell populations being compared. However, this is unlikely because we previously showed that CD21hi and CD21lo transitional B cell subsets from healthy donors produce comparable amounts of IgM, IgG, and IgA in vitro (Suryani et al., 2010). Rather, this defect arose from the inability of PIK3CD GOF transitional B cells to acquire or maintain a plasmablast phenotype and gene signature following in vitro activation. Indeed, PIK3CD GOF transitional B cells were impaired in generating CD38hi CD27hi plasmablasts in vitro. Here, the detected IgM secreted by these cells is likely produced by preplasmablasts that have not yet acquired the phenotype and transcriptional profile of plasmablasts (Avery et al., 2005; Jourdan et al., 2011). The finding

Avery et al.
B cell defects due to hyperactive PI3 kinase

Journal of Experimental Medicine
https://doi.org/10.1084/jem.20180010
that PAX5 was down-regulated less in activated PI3K p110δ GOF human transitional B cells compared with normal B cells may contribute to the altered gene expression profile and impaired Ig secretion by these cells, since loss of PAX5 function is a prerequisite for the differentiation and commitment of B cells to the PC fate (Kallies et al., 2007). Thus, it would be expected that the increased numbers of immature transitional cells and their decreased function, coupled with impaired survival and trafficking due to poor expression of Bcl2 and homeostatic chemokine receptors, would combine to greatly impact the level of functional antibody produced in PI3K GOF patients. Interestingly, our microarray analyses revealed increased IL10 expression by PIK3CD GOF transitional B cells compared with controls. Human transitional B cells have been proposed as being a rich source of IL-10 (Blair et al., 2010). But this is not unique to transitional cells, because naïve, memory and GC B cells can all secrete IL-10 following in vitro stimulation (Burdin et al., 1996; Good et al., 2006). Interestingly, IL-10 is a growth and differentiation factor for human B cells, and endogenous IL-10 contributes to B cell differentiation induced by CD40L/BCR engagement (Moenes and Tangye, 2014). However, despite the ability of IL-10 to promote B cell differentiation, the potential elevated expression of IL10 by PIK3CD GOF transitional B cells was insufficient to rescue the defect in CD40L/IL-21–mediated differentiation of these cells in vitro.

The PI3K/AKT/mTOR pathway is activated in 40–50% of cases of diffuse large B cell lymphoma (Xu et al., 2013). Furthermore, PI3K p110δ inhibitors have been used to treat human B cell malignancies (Hewett et al., 2016; Fruman et al., 2017). Thus, it is possible that PIK3CD GOF mutations intrinsically contribute to B cell lymphomagenesis, which occurs in ~20% of PIK3CD GOF patients (Coulter et al., 2017). However, PI3K inhibitors increase AID expression and activity in murine and human B cells, causing increased genomic instability in vitro and lymphomagenesis in vivo (Compagno et al., 2017). Our finding of reduced levels of AID in PI3K p110δ GOF B cells could infer that PIK3CD activating mutations are not directly oncogenic. Rather, B cell lymphoma may result from impaired effector function of PIK3CD GOF NK and CD8+ T cells (Edwards et al., 2018; Ruiz-García et al., 2018).

The etiology and mechanisms underlying lymphoma development in PIK3CD GOF patients is an area requiring further investigation. Determining whether PI3K p110δ inhibitors (Rao et al., 2017) influence the incidence of B cell lymphoma may provide valuable insight into these outstanding questions. Collectively, these data provide significant insight into the pathological mechanisms underlying hyper-IgM, hypogammaglobulinemia, and impaired long-lived humoral immune responses in PIK3CD GOF patients (Jou et al., 2006; Angulo et al., 2013; Lucas et al., 2014a; Elgizouli et al., 2016; Coulter et al., 2017). Our data also identify putative regulatory networks in B cells that are controlled by PI3K signaling and may play a role in PC differentiation. Importantly, the impairment in secretion of switched Ig isotypes by human PIK3CD GOF transitional B cells, and in class switching and Aicda expression by Pik3cdE1020K GOF murine B cells, could be greatly improved by the PI3K p110δ inhibitor leniolisib. Based on these results leniolisib may improve the ability of patients to generate effective switched Ab responses, eventually resulting in a requirement for Ab replacement therapy, which is currently used in >80% of patients (Coulter et al., 2017). The efficacy of leniolisib in treating PIK3CD GOF patients (Rao et al., 2017), and our demonstration that it improves B cell function in vitro, suggests p110δ inhibitors could be suitable therapeutics for other immune dysregulatory conditions due to defects in PI3K signaling. These include individuals not only with PIK3CD GOF mutations, but also those with inactivating mutations in PIK3R1 (Deau et al., 2014; Lucas et al., 2014b; Elgizouli et al., 2016) or PTEN (Driessen et al., 2016; Tsujita et al., 2016), or even patients with clinical features overlapping these conditions but with hitherto unknown genetic diagnoses (Deenick et al., 2018). However, such treatment will need to be monitored carefully to mitigate the potential risk of accelerating or exacerbating lymphoma development in these patients.

Overall, our detailed analysis has provided insight into the pathophysiology of disease due to PIK3CD GOF mutations. Furthermore, our observations that the Pik3cdE1020K GOF mutation in murine B cells mirrored the effects of PIK3CD GOF mutations in humans underscores the utility of this mouse model to investigate the consequences of hyperactive PI3K signaling and to dissect mechanisms of disease pathogenesis in humans with corresponding mutations. Thus, these mice represent a valuable preclinical model to screen new or alternative pharmacological or biological inhibitors of the PI3K pathway as novel therapeutics for humans with PIK3CD GOF mutations.

Materials and methods

Human blood and bone marrow samples

Buffy coats from healthy donors were purchased from the Australian Red Cross Blood Service. Pediatric blood samples were collected from individuals either attending clinic for nonimmunological conditions, or for genetic testing due to a family history of disease, but were found to be mutation negative. BM was obtained from individuals undergoing lymphoma staging, and was found to be uninvolved. Peripheral blood and BM samples were also collected from patients with GOF mutations in PIK3CD. Human tonsils were obtained from healthy donors undergoing routine tonsillectomy (Mater Hospital, North Sydney). Approval for this study was obtained from the human research ethics committees of St. Vincent’s Hospital (Sydney, Australia), Sydney South West Area Health Service (Sydney, Australia), Royal Children’s Hospital Melbourne (Melbourne, Australia), and the National Institute of Allergy and Infectious Diseases Intramural Institutional Review Board (Bethesda, MD); informed consent was obtained from all participants for human experiments described in this study.

Human antibodies

The following mAbs were used: FITC-anti CD20, PE-Cy7-anti-CD27, APC-anti-CD10, PE-anti-CD5, APC-anti-CD38, BV421-anti-CD21, biotin anti-CD44, BV711-anti-CD19, FITC anti-CCR7 (CD197), PE anti-CXCR4 (CD184), biotin anti-CXCR5 (CD185), PE-anti-IgM, BV605-anti-IgG, biotin-anti-IgA, PE-anti-CD8-PE, PE-anti-Blimp-1, Streptavidin-BV605, and Streptavidin-PerCP-Cy5.5 (all from Becton Dickinson); PE-anti-CD23 and FITC-anti-CD38 (eBioscience); and biotin-anti-IgD (Southern Biotech).

Avery et al.

B cell defects due to hyperactive PI3 kinase

Journal of Experimental Medicine

https://doi.org/10.1084/jem.20180010
PBMCs were incubated with mAbs against CD20, CD27, and CD10 with mAb specific for CD5, CD38, CD21, CD23, CD44, CD19, CCR7, CXCR4, CXCR5, and Bcl-2. The proportions of CD20^+CD27^− (transitional), CD20^+CD27^− (naive), and CD20^+CD27^+ (memory) B cells, as well as the expression of these molecules on these subsets, was determined by flow cytometry (LSRII SORP; Becton Dickinson) and analyzed using FlowJo software (Tree Star; Cuss et al., 2006; Suryani et al., 2010). BM aspirates were incubated with mAbs against CD34, CD19, CD20, CD10, IgM, and CD27. Populations of pro-B (CD19^+CD34^+CD10^−CD20^−IgM^−), pre-B (CD19^+CD34^+CD10^−CD20^−IgM^+), immature (CD19^+CD34^−CD10^+IgM^+), and recirculating mature (CD19^−CD34^−CD10^+CD20^−IgM^+), and transitional (CD20^+CD10^+CD27^−) or naive (CD20^+CD10^−CD27^−) B cells were then sorted using a FACSAria II systems (Becton Dickinson; Cuss et al., 2006; Suryani et al., 2010). Purity of the recovered populations was >98%. Transitional and naive B cells were then cultured in 96-well U-bottom plates (Falcon; 20–40 × 10^3/200 µl well) for 5–7 d to determine proliferation and gene expression by qPCR and microarray analysis, or 5 × 10^4/200 µl well for 5–7 d to determine Ig secretion. B cells were incubated with 200 ng/ml CD40L cross-linked to 50 ng/ml HA Peptide mAb (R&D Systems) alone or together with 100 U/ml IL-4, 100 U/ml IL-10 (provided by R. de Waal Malefyt, DNA Research Institute, Palo Alto, CA), 50 ng/ml IL-21 (PeproTech), 2.5 µg/ml F(ab')2 fragment of goat anti-IgA/IgG/IgM (H+L; Jackson ImmunoResearch), 1 µg/ml CpG 2006 (Sigma-Aldrich), and varying doses of the PI3K p110δ specific inhibitor leniolisib (Hoegenauer et al., 2017; Rao et al., 2017; Novartis Pharma), and equivalent doses of DMSO.

Analysis of human B cell Differentiation in vitro.
B cell viability was determined using the Zombie Aqua Viability dye (BioLegend) and proliferation determined by CFSE (eBioscience) dilution after 4–5 d of in vivo culture (Avery et al., 2010). Differentiation of B cells to plasmablasts was assessed by determining the frequency of transitional or naive B cells acquiring a CD38^hi CD27^hi phenotype during in vitro culture (Avery et al., 2005, 2010). To determine expression of intracellular Ig and Blimp-1, cells were initially labeled with mAbs to CD27 and CD28, then fixed and permeabilized (Foxp3/Transcription Factor Staining Buffer; eBioscience). Permeabilized cells were then incubated with a cocktail of mAbs to IgG, IgM, IgA, and Blimp-1 and expression of these proteins in plasmablasts was determined by flow cytometry (LSRII SORP; Becton Dickinson) and analyzed using FlowJo software (Tree Star).

qRT-PCR
Total RNA was isolated from unstimulated FACS-sorted transitional and naive B cells from normal donors or PIK3CD GOF patients using an RNeasy Mini Kit (QIAGEN). PCR primers (Integrated DNA Technologies) were designed using the Roche UPL primer design program. Primer sequences and Roche UPL probes are as follows: GAPDH, UPL probe 60, forward 5′-CCTGTGCTCC TCTCGTGCAG-3′; reverse 5′-AGCACAAATCCGGTGA CACT-3′; RPL13A (Ribosomal Protein L13a), UPL probe 28, forward 5′-CAA GCGGTAAACCAATCAAC-3′, reverse 5′-TGGGGGCGAGCATCACC-3′, LIF1 (Lymphoid enhancing binding factor–1), UPL probe 17, forward 5′-CAGATGTAACCTCAACAAACAGG-3′, reverse 5′-GGA GCAAGGGATAAAGGAGGG-3′; DTXI (Deltex-1), UPL probe 57, forward 5′-GGGCGAACAACCTAAC-3′, reverse 5′-CAAGTT CTTCAACGCGGATG-3′ (Suryani et al., 2010).

To assess gene expression in activated B cells, total RNA was isolated from B cells cultured with CD40L ± IL-21 for 4–5 d. Primer sequences and Roche UPL probes were: PRDM1 (PR Domain Zinc Finger Protein 1), UPL probe 67, forward 5′-AGTGTGCGGTACGAC TTG-3′, reverse 5′-TGGCAATCCTCAGGACC-3′; AICDA (Activation-Induced Cytidine Deaminase), UPL probe 69, forward 5′-GAC TTTGTATGCTCAGGAAATAA-3′, reverse 5′-GAGTCCGATCC GGAGAT-GTA-3′; MSA47, UPL probe 31, forward 5′-GATCCGATTA TGCACAACCTAC-3′; CD27, UPL probe 6, forward 5′-TTGACTAATTTCCCAGGCTACG-3′, reverse 5′-CACGAAAGTGCTAAGTCTGCTG-3′; ZNF215, UPL probe 82, forward 5′-TTAAAGTGAAAAATGTA ACAA-3′, reverse 5′-GCTCCCTCTACAGGCACACAAG-3′; MYO1D, UPL probe 2, forward 5′-TCTCTCAACCTTGAAGAGTCA-3′, reverse 5′-AGAAAGTCTCGAGTATTGTCG-3′; KMO, UPL probe 90, forward 5′-AAAAAGTTCTGGTGGTATCATCCT-3′, reverse 5′-CCATCGACCTTATCCCTTTCTC-3′; SLAMP7, UPL probe 67, forward 5′-TTAGGTCTTCAGATGCTC-3′, reverse 5′-CTCCTACTATGG TGGGATATAGCTCA-3′; BEX5, UPL probe 44, forward 5′-GCA CAACAGGCTTTAAGACCA-3′, reverse 5′-CCTGCTGTTGGCCTG AAG-3′; RAP1GAP2, UPL probe 38, forward 5′-GCGTCTATGAC GTCTTCAG-3′, reverse 5′-TGATCCTGTTGCTCAAGTG-3′; CD27, UPL probe 30, forward 5′-GGCTCCAGACATCTACC-3′, reverse 5′-CCTACCCTCCCCACTTCAAT-3′; MS4A47, UPL probe 31, forward 5′-GGTACGTAATGGCACAAA-3′, reverse 5′-TCCAACCTCTTC GCTGAC-3′. All amplification reactions were performed using the Roche LightCycler 480 Probe Master Mix and System (Roche Diagnostics) with the following conditions: denaturation at 95°C for 10 min; amplification, 45 cycles at 95°C for 10 s, 65°C for 30 s, and 72°C for 30 s.

Microarray analysis
RNA was extracted from transitional and naive B cells, cultured for 4 d with CD40L alone or together with IL-21, and transcribed into cDNA. Raw gene expression data were obtained using the human Clarion S Assay microarray platform. All preprocessing and analyses were performed using Bioconductor packages implemented in the R statistical computing environment, version 3.4.1. The datasets were merged and batch corrected using ComBat of the sva package. Differential gene expression analysis was assessed using limma. Heat maps were generated using the heatmap package. Unsupervised dimension reduction and metagene extraction was performed with the NMF package, as
RNA-seq analysis

Naive, memory, GC, and plasma cells were sorted from tonsils from healthy donors and subjected to RNA sequencing. RNA-seq reads were aligned to the GRCm38/hg19 build of the human genome using the Subread aligner (Liao et al., 2013). Geneewise counts were obtained using featureCounts (Liao et al., 2014). Reads overlapping exons in annotation build 37.2 of NCBI RefSeq database were included. Genes were excluded from downstream analysis if they failed to achieve a CPM (counts per million mapped reads) value of 1 or greater in at least three libraries. Counts were converted to log2-CPM, quantile normalized and precision weighted with the voom function of the limma package (Law et al., 2014; Ritchie et al., 2015). A linear model was fitted to each gene, and empirical Bayes moderated t-statistics were used to assess differences in expression (Smyth, 2004).

Human Ig ELISAs

Secretion of IgM, IgG and IgA by in vitro cultured human transitional and naive B cells was determined using Ig heavy-chain specific ELISAs, as described previously (Avery et al., 2005, 2010).

Mice

Pik3cdE1020K GOF mice were produced by the Mouse Engineering Garvan/ABR (MEGA) Facility using CRISPR/Cas9 gene targeting in C57BL/6 mouse embryos following established molecular and animal husbandry techniques (Yang et al., 2014). The single guide RNA (sgRNA) was based on a target site in the final exon of Pik3cd (GAG) and was associated with the CRISPR/Cas9 gene targeting system. The sgRNA was then microinjected into the nucleus and cytoplasm of C57BL/6 jygote together with polyadenylated Streptococcus pyogenes Cas9 mRNA and a 66 base single-stranded, anti-sense, deoxy-oxygenucleotide homologous recombination substrate carrying the EI020K (GAA>AAA) mutation and a PAM-inactivating silent mutation in the F1014 codon (TTG>TTC; underlined nucleotides indicate those targeted by CRISPR and the subsequent change). A male founder mouse heterozygous for both substitutions was obtained and backcrossed with C57BL/6 female mice to establish the Pik3cdE1020K GOF line. For some experiments Pik3cdE1020K GOF mice were then crossed with SWHEL mice (Brink et al., 2008, 2015) to generate SWHEl.Pik3cdE1020K GOF mice. Donor cells were adoptively transferred into C57/Bl/6 (CD45.1 congenic) mice purchased from Australian BioResources. Donor-derived IgG1 switched GC B cells were identified using anti-IgM (R6-60.2), anti-IgG1 [A85-1], biotinylated anti-IgG2a/c [R19-15], biotinylated anti-IgG2b [R12-3], biotinylated anti-IgG3 [R40-82], and biotinylated anti-IgM (R6-60.2). Ig levels for each class were quantified against recombinant HyHEL10 standards (Brink et al., 2015).

Single-cell sorting and SHM analysis

Recipient spleens were prepared and stained for flow cytometry. Donor derived IgG1 switched GC B cells were identified using anti-CD45.2 PE/Cy7, anti-CD23 PerCP Cy5.5, anti-B220 PE, and anti-CD38 FITC, anti-IgG1-biotin, and SA-PB. Single cells were sorted using FACSARia III (Becton Dickinson). The variable region exon of the SWHEl Ig (HyHEL10) heavy chain regions was amplified by PCR and sequenced. The final product was sequenced by GEN EWIZ and analyzed.
Flow cytometry of mouse cells
The following were purchased from BD Biosciences: anti-CD45/R/B220 FITC, PE, BV786 and PerCp Cy5.5, anti-CD24 PE (M1/69), anti-CD19 BV510 (ID3), biotinylated anti-CD43 (S7), biotinylated anti-IgG1 (A85-1), biotinylated anti-IgG2a/c (R19-15), biotinylated anti-IgG2b (R12-3), biotinylated anti-IgG3 (R40-82), biotinylated anti-IgM (AF6-78), anti-CD16/CD32 Fc block (2.4G2), Streptavidin-BV605, Streptavidin-BV711, and Streptavidin-BUV395. The following were purchased from eBiosciences: anti-CD5 APC (53–73), anti-CD38 FITC (90), anti-CD45.2 PerCP-Cy5.5 (104), anti-CD45.1 PeCy7 (A20), anti-CD23 PeCy7 (B384), biotinylated anti-CD93 (AA4.1). The following was purchased from Invitrogen: Streptavidin-PB.

The following was purchased from Life Technologies: Streptavidin-PE, Streptavidin-APC, Streptavidin-BUVA, Streptavidin-BV420, Streptavidin-BV605, Streptavidin-BV711, and Streptavidin-BV785. The following were purchased from eBiosciences: anti-CD5 APC (53–73), anti-CD38 FITC (90), anti-CD45.2 PerCP-Cy5.5 (104), anti-CD45.1 PeCy7 (A20), anti-CD23 PeCy7 (B384), biotinylated anti-CD93 (AA4.1).

BM and spleens of WT or Pik3cdE1020K GOF mice, and spleens of recipient mice in adoptive transfer studies were harvested at the indicated time points, prepared and stained for flow cytometry as previously described (Chan et al., 2009). For intracellular staining of phospho-Akt or phospho-S6 cells were stained directly ex vivo for B220 then fixed in 2% formaldehyde, permeabilized in 90% methanol, and labeled with anti-phosphospecific antibodies (Avery et al., 2010). Data were acquired on either (LSR II) SORP or FOR 10ES SA; Becton Dickinson) and analyzed using FlowJo software (Tree Star). All data are representative of two or more experiments as indicated.

Statistical analysis
Significant differences were determined using Prism (GraphPad Software).

Online supplemental information
Fig. S1 shows increased phosphorylation of S6 in total B cells, increased expression of DTX and LEF1 in transitional and naive B cells from patients with PIK3CD GOF mutations (red histograms/graphs) compared healthy donors (black histograms/graphs), and intact proliferation in vitro of control (black histograms) and PIK3CD GOF (red histograms) naive B cells in response to various stimuli. Fig. S2 shows differentially expressed genes in activated transitional B cells from healthy donors and patients with PIK3CD GOF mutations, differential expression of the indicated genes in human naive, memory, GC, and PC isolated from tonsils obtained from healthy donors, as determined by RNA Seq; differential expression of Siglec-6, CRACC, and CD27 on the surface of human tonsillar naive, memory, GC, and PC, as determined by immunofluorescent staining and flow cytometric analysis; differential expression of Siglec-6, CRACC, and CD27 on the surface of activated (i.e., CD20+CD28+) B cells and plasmablasts (i.e., CD20hiCD38hi), present in cultures of naive B cells from healthy donors cultured with CD40L/IL-21. Fig. S3 depicts increased expression of pAKT and pS6 in Pik3ce1020K GOF murine B cells and correction of elevated levels by the p110δ inhibitor leniolisib, and proportions of total, B1, transitional, and mature B cells in the blood of WT and Pik3ce1020K GOF mice.

References
Aagaard-Tillery, K.M., and D.F. Jelinek. 1996. Phosphatidylinositol 3-kinase activation in normal human B lymphocytes. J. Immunol. 156:4543–4554.
Agematsu, K., H. Nagumo, F.C. Yang, T. Nakazawa, K. Fukushima, S. Ito, K. Sugita, T. Mori, T. Kobata, C. Morimoto, and A. Komiyama. 1997. B cell activation in normal human B lymphocytes. J. Immunol. 158:3860–3867. https://doi.org/10.4049/jimmunol.158.7.3860
Andjelic, S., C. Hsia, H. Suzuki, T. Kadowaki, S. Koyasu, and H.C. Liou. 2000. Phosphatidylinositol 3-kinase and NF-kappa B/Rel are at the divergence of CD40-mediated proliferation and survival pathways. J. Immunol. 165:3860–3867. https://doi.org/10.4049/jimmunol.165.7.3860
Angulo, I., O. Vadas, F. Garçon, E. Banham-Hall, V. Plagnol, T.R. Leahy, H. Baxendale, T. Coulter, J. Curtis, C. Wu, et al. 2013. Phosphoinositide 3-ki-
Avery et al.

B cell defects due to hyperactive PI3 kinase

Journal of Experimental Medicine

https://doi.org/10.1084/jem.20180010

Sander, S., V.T. Chu, T. Yasuda, A. Franklin, R. Graf, D.P. Calado, S. Li, K. Imami, M. Selbach, M. Di Virgilio, et al. 2015. PI3 Kinase and FOXO1 Transcription Factor Activity Differentially Control B Cells in the Germinal Center Light and Dark Zones. *Immunity.* 43:1075–1086. https://doi.org/10.1016/j.immuni.2015.10.021

Sims, G.P., R. Ettinger, Y. Shirota, C.H. Yarboro, G.G. Illei, and P.E. Lipsky. 2005. Identification and characterization of circulating human transitional B cells. *Blood.* 105:4390–4398. https://doi.org/10.1182/blood-2004-11-4284

Smyth, G.K. 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* 3:e3. https://doi.org/10.2202/1544-6115.1027

Srinivasan, L., Y. Sasaki, D.P. Calado, B. Zhang, J.H. Paik, R.A. DePinho, J.L. Kutok, J.F. Kearney, K.L. Otipoby, and K. Rajewsky. 2009. PI3 kinase signals BCR-dependent mature B cell survival. *Cell.* 139:573–586. https://doi.org/10.1016/j.cell.2009.08.041

Suryani, S., D.A. Fulcher, B. Santner-Nanan, R. Nanan, M. Wong, P.J. Shaw, J. Gibson, A. Williams, and S.G. Tangye. 2010. Differential expression of CD21 identifies developmentally and functionally distinct subsets of human transitional B cells. *Blood.* 115:519–529. https://doi.org/10.1182/blood-2009-08-24999

Suzuki, A., T. Kaisho, M. Ohishi, M. Tsukio-Yamaguchi, T. Tsubata, P.A. Koni, T. Sasaki, T.W. Mak, and T. Nakano. 2003. Critical roles of Pten in B cell homeostasis and immunoglobulin class switch recombination. *J. Exp. Med.* 197:657–667. https://doi.org/10.1084/jem.20021101

Suzuki, H., Y. Terauchi, M. Fujiwara, S. Aizawa, Y. Yazaki, T. Kadowaki, and S. Koyasu. 1999. Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. *Science.* 283:390–392. https://doi.org/10.1126/science.283.5400.390

Tang, P., J.E.M. Upton, M.A. Barton-Forbes, M.I. Salvadori, M.P. Clynick, A.K. Price, and S.L. Goobie. 2018. Autosomal Recessive Agammaglobulinemia Due to a Homozygous Mutation in PIK3R1. *J. Clin. Immunol.* 38:88–95. https://doi.org/10.1007/s10875-017-0462-y

Tangye, S.G., C.S. Ma, R. Brink, and E.K. Deenick. 2013. The good, the bad and the ugly - TFH cells in human health and disease. *Nat. Rev. Immunol.* 13:412–426. https://doi.org/10.1038/nri3447

Tangye, S.G., R. Brink, C.C. Goodnow, and T.G. Phan. 2015. SnapShot: Interactions between B Cells and T Cells. *Cell.* 162:926–6.e1. https://doi.org/10.1016/j.cell.2015.07.055

Tsujita, Y., K. Mitsui-Sekinaka, K. Imai, T.W. Yeh, N. Mitsuiuki, T. Asano, H. Ohnishi, Z. Kato, Y. Sekinaka, K. Zaha, et al. 2016. Phosphatase and tensin homolog (PTEN) mutation can cause activated phosphatidylinositol 3-kinase δ syndrome-like immunodeficiency. *J. Allergy Clin. Immunol.* 138:1672–1680.e10. https://doi.org/10.1016/j.jaci.2016.03.055

Uckun, F.M. 1990. Regulation of human B-cell ontogeny. *Blood.* 76:1908–1923.

van Zelm, M.C., M. van der Burg, D. de Ridder, B.H. Barendregt, E.F. de Haas, M.J. Reinders, A.C. Lankester, T. Révész, F.J. Staal, and J.J. van Dongen. 2005. Ig gene rearrangement steps are initiated in early human precursor B cell subsets and correlate with specific transcription factor expression. *J. Immunol.* 175:5912–5922. https://doi.org/10.4049/jimmunol.175.9.5912

Xu, Z.Z., Z.G. Xia, A.H. Wang, W.F. Wang, Z.Y. Liu, L.Y. Chen, and J.M. Li. 2013. Activation of the PI3K/AKT/mTOR pathway in diffuse large B cell lymphoma: clinical significance and inhibitory effect of rituximab. *Ann. Hematol.* 92:1351–1358. https://doi.org/10.1007/s00277-013-1770-9

Yang, H., H. Wang, and R. Jaenisch. 2014. Generating genetically modified mice using CRISPR/Cas-mediated genome engineering. *Nat. Protoc.* 9:1956–1968. https://doi.org/10.1038/nprot.2014.134

Zeng, R., R. Spolski, E. Casas, W. Zhu, D.E. Levy, and W.J. Leonard. 2007. The molecular basis of IL-21-mediated proliferation. *Blood.* 109:4135–4142. https://doi.org/10.1182/blood-2006-10-054973
Author/s:
Avery, DT; Kane, A; Nguyen, T; Lau, A; Nguyen, A; Lenthall, H; Payne, K; Shi, W; Brigden, H; French, E; Bier, J; Hermes, JR; Zahra, D; Sewell, WA; Butt, D; Elliott, M; Boztug, K; Meyts, I; Choo, S; Hsu, P; Wong, M; Berglund, LJ; Gray, P; O'Sullivan, M; Cole, T; Holland, SM; Ma, CS; Burkhart, C; Corcoran, LM; Phan, TG; Brink, R; Uzel, G; Deenick, EK; Tangye, SG

Title:
Germline-activating mutations in PIK3CD compromise B cell development and function

Date:
2018-08-01

Citation:
Avery, D. T., Kane, A., Nguyen, T., Lau, A., Nguyen, A., Lenthall, H., Payne, K., Shi, W., Brigden, H., French, E., Bier, J., Hermes, J. R., Zahra, D., Sewell, W. A., Butt, D., Elliott, M., Boztug, K., Meyts, I., Choo, S., ... Tangye, S. G. (2018). Germline-activating mutations in PIK3CD compromise B cell development and function. JOURNAL OF EXPERIMENTAL MEDICINE, 215 (8), pp.2073-2095. https://doi.org/10.1084/jem.20180010.

Persistent Link:
http://hdl.handle.net/11343/251142

File Description:
published version

License:
CC BY-NC-SA