Hyperactivated PI3Kδ promotes self and commensal reactivity at the expense of optimal humoral immunity

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Gain-of-function mutations in the gene encoding the phosphatidylinositol-3-OH kinase catalytic subunit p110δ (PI3Kδ) result in a human primary immunodeficiency characterized by lymphoproliferation, respiratory infections and inefficient responses to vaccines. However, what promotes these immunological disturbances at the cellular and molecular level remains unknown. We generated a mouse model that recapitulated major features of this disease and used this model and patient samples to probe how hyperactive PI3Kδ fosters aberrant humoral immunity. We found that mutant PI3Kδ led to co-stimulatory receptor ICOS-independent increases in the abundance of follicular helper T cells (Tfh cells) and germinal-center (GC) B cells, disorganized GCs and poor class-switched antigen-specific responses to immunization, associated with altered regulation of the transcription factor FOXO1 and pro-apoptotic and anti-apoptotic members of the BCL-2 family. Notably, aberrant responses were accompanied by increased reactivity to gut bacteria and a broad increase in autoantibodies that were dependent on stimulation by commensal microbes. Our findings suggest that proper regulation of PI3Kδ is critical for ensuring optimal host-protective humoral immunity despite tonic stimulation from the commensal microbiome.

PI110δ, a catalytic subunit of phosphatidylinositol-3-OH kinase (PI3K) expressed mainly in hematopoietic cells, is activated by cytokine, antigen and co-stimulatory receptors and coordinates signaling involved in the activation and differentiation of T cells and B cells1. Patients with gain-of-function point mutations in the gene encoding p110δ (PIK3CD) exhibit the primary immunodeficiency PASLI (‘p110δ-activating mutation causing senescent T cells, lymphadenopathy and immunodeficiency’), also known as APDS (‘activated-PI3Kδ syndrome’; called ‘APDS’ here), characterized by lymphopenia, lymphoproliferation, recurrent respiratory infections and mucosal lymphoid follicles. Such patients display an increased abundance of effector T cells and a reduced abundance of naive T cells, enlarged germinal centers (GCs), fewer class-switched memory B cells, and impaired antibody responses to vaccination2-4. However, the cellular and molecular events that contribute to these phenotypes remain to be characterized.

Clues to how altered activity of PI3K p110δ (PI3Kδ) might disrupt antibody responses have been provided by work demonstrating that T cells and B cells intimately act together in antigen-driven antibody responses via the generation of GCs, which are specialized microenvironments for immunoglobulin class switching, affinity maturation and the development of memory B cells and long-lived plasma cells5. GCs also help maintain tolerance through the elimination of self-reactive clones6. CD4+ follicular helper T cells (Tfh cells) provide essential signals for the formation and maintenance of GCs, as well as for the survival and selection of B cells that produce high-affinity antibodies7,8 and the deletion of potentially autoreactive B cells9. Tfh cells express the chemokine receptor CXCR5, the inhibitory receptor PD-1, the co-stimulatory receptor ICOS and the transcription factor BCL-610. In activated T cells, ICOS potently activates PI3Kδ, which leads to inactivation of FOXO1, a transcriptional repressor of Bcl611,12. In B cells, PI3Kδ is critical for their survival, proliferation and differentiation, acting via the integration of signals from the B cell antigen-receptor (BCR), the co-stimulatory receptors CD19 and CD40, the receptor for the B cell–stimulatory molecule BAFF, cytokines and Toll-like-receptors (TLRs)13. Notably, mice that lack PI3Kδ activity have defective formation of Tfh cells and GCs14,15, which demonstrates important roles for PI3Kδ in GC reactions. Furthermore, almost half of patients with APDS display autoantibodies and autoimmune-mediated organ damage16,17, which

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Articles

NaTure ImmuNOLOGY

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NaTure ImmuNOLOGY (left), frequency of PD-1a generation of hyperactivated immunophenotypes. Our results suggested that regulation of PI3Kδ activity is also important for limiting self-reactivity. These findings raise the possibility that GCs might be a major site of aberrant activity by lymphocytes expressing mutant PI3Kδ.

Here we generated a mouse model of APDS (Pik3cd<sup>E1020K</sup>/+ mice) and used this model to study how overactive PI3Kδ (PI3Kδ<sup>E1020K</sup>) affects humoral responses, focusing on GCs. We found that PI3Kδ<sup>E1020K</sup> led to exaggerated production of T<sub>FH</sub> cells and GCs, associated with lymphocyte hyperactivity, altered regulation of FOXO1 and a member of the BCL-2 family, and increased B cell survival. Nonetheless, Pik3cd<sup>E1020K</sup>/− mice displayed a diminished capacity for the production of robust class-switched antigen-specific antibodies in response to immunization, despite augmented anti-self and anti-commensal responses. Notably, treatment of Pik3cd<sup>E1020K</sup>/− mice with antibiotics revealed a requirement for commensal stimulation in the generation of hyperactivated immunophenotypes. Our results suggest that tight regulation of PI3Kδ activity is critical for constraining tonic activation induced by the commensal microbiome while facilitating protective immune responses.

Fig. 1 Greater abundance of circulating T<sub>FH</sub> cells in patients with APDS. a. Flow-cytometry analysis of the expression of the naive-cell marker CD45RA and chemokine receptor CXCR5 by CD4<sup>+</sup>CD3<sup>+</sup>CD25<sup>−</sup>-T cells from the blood from healthy donors (HD) and patients with APDS (above plots) (left), and frequency of CXCR5<sup>−</sup>cT<sub>FH</sub> cells among CD4<sup>+</sup>CD3<sup>+</sup>CD25<sup>−</sup>-T cells from healthy donors (n = 8) and patients with APDS (n = 7) as at left (right). Numbers adjacent to outlined areas (left) indicate percent CD45RA<sup>−</sup>CXCR5<sup>−</sup>-T cells. b. Flow-cytometry analysis of the expression of PD-1 and CXCR5 by T cells as in a (left), frequency of PD-1<sup>+</sup>CXCR5<sup>−</sup>-T cells (middle) and the geometric mean fluorescent intensity (gMFI) of PD-1 on PD-1<sup>+</sup>-cT<sub>FH</sub> cells (right). Numbers adjacent to outlined areas (left) indicate percent PD-1<sup>+</sup>CXCR5<sup>−</sup>-T cells. c. Flow-cytometry analysis of the expression of the chemokine receptors CCR6 and CXCR3 by T cells as in a (left), frequency of CXCR3<sup>+</sup>cT<sub>FH</sub> cells (middle) and gMFI of CXCR3 on CXCR3<sup>+</sup>cT<sub>FH</sub> cells (right). Numbers in quadrants (left) indicate percent cells in each. Each symbol represents an individual donor or patient; small horizontal lines indicate the mean (± s.e.m.). *P < 0.05, **P < 0.01 and ***P < 0.001 (Mann-Whitney U-test).

Suggestions

Patients with APDS display an increased abundance of blood T<sub>FH</sub> cells. Circulating T<sub>FH</sub> cells (cT<sub>FH</sub>) increase in abundance after immunization and in autoimmune conditions and reflect true T<sub>FH</sub> cells residing in secondary lymphoid organs<sup>17</sup>. Despite their impaired antigen-specific responses<sup>16</sup>, we found a greater frequency of CD4<sup>+</sup>CD45RA<sup>−</sup>CD25<sup>−</sup>-CXCR5<sup>−</sup>cT<sub>FH</sub> cells in patients with APDS than in healthy donors (Fig. 1a). The cT<sub>FH</sub> cells from the patients also exhibited elevated expression of PD-1 (Fig. 1b), which is associated with enhanced effector phenotypes and ongoing GC reactions<sup>18</sup>. Furthermore, the patients exhibited a greater frequency of CXCR3<sup>−</sup>cT<sub>FH</sub> cells than that of healthy donors (Fig. 1c), consistent with a bias toward T<sub>FH</sub>1 cells, which do not effectively help naive B cells in vitro<sup>17</sup>. Thus, the patients with APDS showed altered homeostasis of effector CD4<sup>+</sup>-T cell populations that influence humoral responses.

Pik3cd<sup>E1020K</sup>/− mice recapitulate features of APDS. To explore the effect of hyperactivated PI3Kδ on immune responses, we generated a mouse model that expressed p110<sup>δ</sup>E1021K in patients with APDS<sup>14</sup> (Supplementary Fig. 1a). Heterozygous Pik3cd<sup>E1020K</sup>/− mice recapitulated many features of APDS<sup>14</sup>, including fewer circulating white blood cells (Fig. 2a), lymphadenopathy (Supplementary Fig. 1b) and increased splenic cellularity (Fig. 2b), relative to that of wild-type mice.

The most common clinical phenotype of patients with APDS is recurrent respiratory infections, often associated with lung and tracheal mucosal nodules<sup>14</sup>. Additionally, ~30% of the patients display enteropathy with gastrointestinal nodular mucosal lymphoid hyperplasia<sup>14</sup>. Similar to results obtained for patients, we found evidence of perivascular and peribronchiolar lymphoid aggregates in the lungs of the mutant mice (Fig. 2c, left) and an increased number and size of isolated lymphoid follicles in the small intestines of the mutant mice, relative to those of wild-type mice (Fig. 2c, right). These similarities suggested that Pik3cd<sup>E1020K</sup>/− mice would provide a useful tool with which to delineate the cellular and molecular mechanisms that contribute to the corresponding human disease.

To evaluate how PI3Kδ specifically affects lymphocytes, we assessed T cell and B cell populations. Despite the similar frequency of CD4<sup>+</sup>-T cells in mutant and wild-type mice, we found a lower frequency of naive CD4<sup>+</sup>-T cells and a greater frequency of activated CD4<sup>+</sup>-T cells in the spleen and peripheral lymph nodes (LNs) of mutant mice than in those of wild-type mice (Fig. 2d and Supplementary Table 1),
similar to results obtained for patients’ blood\(^{24}\). These phenotypes became more pronounced over time (Supplementary Table 1); by 1 year of age, Pik3cd\(^{E1020K/+}\) mice had almost no CD62L:\^\text{CD44}^\text{lo} naive CD4^+ T cells (Supplementary Fig. 1c).

A partial block in the development of B cells is seen in patients with APDS, with increased circulating transitional CD20^+CD10^+ B cells\(^{19}\). Although the frequency of splenic B cells in mutant mice was similar to that of wild-type mice (Supplementary Table 1),
mutant mice had a greater number of and altered populations of CD93+ transitional B cells, including more T1 cells, consistent with a partial developmental block (Supplementary Fig. 1d). Additionally, mutant mice had altered surface expression of immunoglobulin M (IgM) and IgD, fewer follicular B cells and more marginal-zone B cells, relative to that of wild-type mice (Supplementary Fig. 1e,f), in support of the proposal of a role for PI3Kδ in driving the differentiation of marginal-zone B cells22. Finally, mutant mice had more peritoneal B-1a cells (Supplementary Fig. 1g,h), a self-reactive and/or innate-like population21. Therefore, Pik3cdΔE1020K/+ mice developed T cells and B cells but exhibited alterations in the activation and homeostasis of lymphocytes, as seen in the corresponding human patients.

**Elevated activation of lymphocytes in Pik3cdΔE1020K/+ mice.** Consistent with observations of patients’ blood, we found a greater frequency of PD-1+CXCR5+CD4+ T cells in the spleen of Pik3cdΔE1020K/+ mice than in that of wild-type mice (Fig. 2e). These included both Foxp3+ Treg cells and Foxp3+ follicular helper regulatory T cells (Tfh cells) (Fig. 2f), yet the ratio of Tfh cells to Treg cells in the mutant mice remained similar to that of wild-type mice (Supplementary Table 1). Both BCL-66+ pre-Tfh cell and BCL-67+ GC Tfh cell populations were expanded (Supplementary Fig. 2a). Furthermore, although 2-month-old mutant mice exhibited a relatively normal number of Tfh cells in the peripheral LNs, the frequency and number of these cells increased in both the LNs and the spleen as mice aged (Supplementary Fig. 2b and Supplementary Table 1). In mutant mice, by 1 year of age, the majority of CD4+ T cells had abundant expression of PD-1, ICOS and CXCR5 (Supplementary Fig. 2c).

In parallel, we detected substantially more GC B cells in both the LNs and the spleen, as well as more splenic CD138+ plasma cells and plasmablasts, in mutant mice than in wild-type mice (Fig. 2g,h and Supplementary Fig. 2d). Consistent with time-dependent increases in lymphocyte activation, these phenotypes became more pronounced in mutant mice with age, together with a reduction in the abundance of IgD+ naive B cells (Supplementary Fig. 2e and Supplementary Table 1). Accordingly, we observed a higher concentration of serum IgM and IgG in the serum of mutant mice than in that of wild-type mice (Fig. 2i). An elevated concentration of serum IgM is also seen in patients with APDS, although IgG concentrations are variable2–4. Thus, like the corresponding patients, Pik3cdΔE1020K/+ mice displayed expanded activated lymphocyte populations associated with increased antibody production.

**Defective T cell–dependent humoral responses.** To explore the effect of activated PI3Kδ on antigen-induced humoral responses, we immunized mice subcutaneously with the T cell–dependent antigen NP-OVA (4-hydroxy-3-nitrophenylacetyl conjugated to ovalbumin) in alum. Mice were immunized at 2 months of age, when the frequency of Tfh cells in mutant mice was relatively normal, or at 4 months of age, when mutant mice exhibited a greater abundance of Tfh cells and GC B cells, similar to the corresponding patients. As expected, after immunization, the frequency of Tfh cells and GC B cells increased in the draining LNs (dLNs) relative to their frequency in the resting LNs (rLNs) in both age groups of wild-type mice (Fig. 3a and Supplementary Fig. 3a). Similarly, the frequency of Tfh cells and GC B cells increased after immunization in young (2-month-old) mutant mice (Supplementary Fig. 3a). In contrast, immunization did not increase the frequency of Tfh cells in GC B cells or GC B cells in the dLNs of 4-month-old mutant mice (Fig. 3a). Furthermore, while at baseline, mutant rLNs had greater cellularity than that of wild-type rLNs, after immunization, mutant dLNs had a number of cells comparable to or even lower than that of wild-type dLNs (Supplementary Fig. 3b).

Despite the greater frequency of GC B cells in mutant mice, the frequency and number of antigen-binding (NP+) GC B cells were lower, so that the ratio of NP+ antigen-specific GC B cells to NP− GC B cells was much lower in mutant mice than in wild-type mice (Fig. 3b,c and Supplementary Fig. 3c). The mean fluorescent intensity of NP-binding cells was also lower in mutant mice than in wild-type mice (Fig. 3b), which might have reflected a lower abundance of BCRs on the surface of mutant cells. These phenotypes became even pronounced by 1 year of age, when many mutant mice had very few NP-specific GC B cells after immunization (Supplementary Fig. 3d). However, a lower ratio of NP+ GC B cells to NP− GC B cells was also observed in 2-month-old mutant mice, relative to that in age-matched wild-type mice (Supplementary Fig. 3e), which suggested that these observations were not solely the result of the prevention of new antigen-specific responses via an increased abundance of pre-existing GCs. Within the NP+ GC B cell compartment, we found a lower frequency of IgG1+ cells in mutant mice than in wild-type mice (Fig. 3d), indicative of impaired class switching in the mutant mice.

Analysis of antibody concentrations in serum revealed a wide range of abundance for NP-specific IgM in Pik3cdΔE1020K/+ mice that overlapped that of wild-type (Fig. 3e). However, consistent with impaired class switching, NP-specific IgG1 was significantly lower in abundance in mutant mice than in wild-type mice (Fig. 3f), despite the finding of greater total IgG1 in the serum of mutant mice, probably a result of an increased abundance of activated B and plasma cells (Supplementary Fig. 3f). Nonetheless, the ratio of high-affinity antibodies to NP, to total antibodies to NP, which is often used to evaluate affinity maturation5, in wild-type mice was similar to that in mutant mice (Fig. 3f). Thus, Pik3cdΔE1020K/+ mice exhibited marked alterations in humoral immunity, characterized by increased basal formation of GCs, but defects in the magnitude of class-switched antigen-specific antibody responses.

**Hyperactivated-Pi3Kδ results in disorganized GCs.** To provide further insight into the nature of the defects reported above, we used high-dimensional immunofluorescence confocal microscopy to evaluate the structure and organization of GCs (Fig. 3g). To model the immune-system activation observed in the corresponding patients, we evaluated 4-month-old mice both at steady state and after immunization. In line with our flow analyses, wild-type rLNs had very few, poorly formed GCs within the follicular dendritic cell (FDC) networks (Fig. 3g, far left); however, after immunization, we observed increased generation of GCs, as indicated by BCL-6+ cells in the dLNs (Fig. 3g, middle left). In contrast, the rLNs of mutant mice already had expanded GC areas that filled the FDC networks (Fig. 3g, middle right). Almost all FDC areas were occupied with BCL-6+ GCs in the dLNs of both wild-type mice and mutant mice, but, strikingly, they were also similarly occupied in the rLNs of mutant mice (Supplementary Fig. 3g).

Enlargement of a wild-type GC permitted identification of the dark zone (DZ), which showed enrichment for tightly packed BCL-6+ GC B cells, and the light zone (LZ), which was demarcated by CD35+ FDCs and included the bulk of the PD-1+CD4+ Tfh cells (Fig. 3g, middle left column, middle and bottom rows). Although the size of mutant dLN GCs varied (Supplementary Fig. 3h), GCs from both mutant rLNs and mutant dLNs showed altered organization, with more invasion of the DZ by Tfh cells (Fig. 3g, middle and far right columns, middle and bottom rows). Those findings were confirmed by histocytometry quantifying Tfh cells per DZ area (Supplementary Fig. 3i–k). Flow cytometry further showed a reduction in the DZ and a greater frequency of LZ NP+ GC B cells in mutant mice than in wild-type mice, as identified by expression of the chemokine receptor CXC4 and the co-stimulatory molecule CD86 (B7-2)13 (Supplementary Fig. 3i). A greater frequency of Foxp3+ regulatory T cells and Tfh cells in mutant mice than in
Fig. 3 | Defective humoral responses and disorganized GCs in Pik3cd<sup>E1020K/+</sup> mice. a, Frequency of PD-1<sup>+</sup>CXCR5<sup>+</sup>Foxp3<sup>+</sup> T<sub>H</sub> cells (among CD4<sup>+</sup>B220<sup>−</sup>T cells) and GL-7<sup>+</sup>FAS<sup>+</sup> GC B cells (among B220<sup>−</sup>CD19<sup>+</sup> B cells) in the popliteal rLN and dLNs (key) of 4-month-old wild-type and Pik3cd<sup>E1020K/+</sup> mice (horizontal axis) immunized subcutaneously with NP-OVA in alum, analyzed on day 10 after immunization. b, Flow-cytometry analysis of the expression of FAS and staining of NP in GC B cells from dLNs as in a (left), frequency of NP antigen-specific (NP<sup>+</sup>) GC B cells in mice as at left (middle), and gMFI of NP on those NP<sup>+</sup> GC B cells (right). c, Ratio of NP<sup>+</sup> GC B cells to NP<sup>−</sup> GC B cells (NP<sup>+</sup>/NP<sup>−</sup>) in dLNs as in a, d, IgG1 staining on NP<sup>+</sup> GC B cells in dLNs as in a (left), and frequency of IgG1<sup>+</sup> NP<sup>+</sup> GC B cells (right). Numbers above bracketed lines (left) indicate percent IgG1<sup>+</sup> cells. e, ELISA of total (anti-NP<sub>20</sub> BSA) IgM in serum from mice as in a, presented in arbitrary units (AU). f, ELISA of high-affinity (anti-NP<sub>4</sub> BSA) and total (anti-NP<sub>20</sub> BSA) NP-specific IgG1 in serum from mice as in a (left and middle), and ratio of high-affinity NP-specific IgG1 to total NP-specific IgG1 (NP<sub>4</sub>/NP<sub>20</sub>) (right). g, Confocal microscopy of the immunofluorescence staining (below images) of frozen sections prepared from popliteal rLN and dLNs as in a (above images): white dashed line demarcates the boundary between the B cell follicle and T cell zone, on the basis of B220 staining (data not shown); LZ, CD35 staining; GCs, BCL-6 staining. Scale bars, 200 µm (top) or 50 µm (middle and bottom). Each symbol (a–f) represents an individual mouse (wild-type, n = 8 (a–c,e,f) or n = 5 (d); Pik3cd<sup>E1020K/+</sup>, n = 6 (a–c,e,f) or n = 6 (d)). Data are representative of two independent experiments (a–f, mean ± s.e.m.) or are from one lymph node representative of two to three independent lymph nodes (g). *P < 0.05, **P < 0.01 and ***P < 0.001 (Mann-Whitney U-test).
wild-type mice was also observed (Fig. 3g, middle and far right), as confirmed by flow cytometry (Supplementary Fig. 3m); however, T_{FH} cells were seen at the T cell–B cell border and within the B cell follicles in both wild-type mice and mutant mice, consistent with a published study\(^2\). Thus, \(\text{Pik3cd}^{E1020K/+}\) mice exhibited disorganized GCs with extensive infiltration of T_{FH} cells into the DZ and poor demarcation of LZ and DZ areas, indicative of a dysregulated immune response.

**Increased T\(_{FH}\) cell differentiation is T cell intrinsic.** The formation of GCs involves intimate interactions and crosstalk between antigen-specific T_{FH} cells and B cells. To probe the distinct cellular contributions to the altered humoral phenotypes, we transferred naive \(\text{Pik3cd}^{E1020K/+}\) OT-II cells (which have transgenic expression of an OVA-specific T cell antigen receptor) into wild-type hosts, which we then immunized intraperitoneally with NP-OVA (Fig. 4a). At 8 d after immunization, \(\text{Pik3cd}^{E1020K/+}\) or \(\text{Pik3cd}^{E1020K/+}\) OT-II cell populations had expanded similarly (Fig. 4b) and were homogeneously CD44\(^+\) (Supplementary Fig. 4a). However, a greater frequency of the \(\text{Pik3cd}^{E1020K/+}\) OT-II cells than \(\text{Pik3cd}^{E1020K/+}\) OT-II cells had acquired the expression of T_{FH} cell markers (Fig. 4c), including markers of both pre-T_{FH} cells and GC T_{FH} cells (Supplementary Fig. 4b). Notably, \(\text{Pik3cd}^{E1020K/+}\) OT-II cell populations produced the cytokine IL-21 and provided in vitro help to B cells similarly to their wild-type counterparts (Supplementary Fig. 4c,d), consistent with normal function. Thus, the \(\text{Pik3cd}^{E1020K/+}\) mutation drove a cell-intrinsic population expansion of T_{FH} cells.

**ICOS-independent generation of T\(_{FH}\) cells.** ICOS is a critical receptor that activates PI3K\(\delta\) and is essential for T_{FH} cell differentiation\(^1\). Since p110\(\delta\)\(^{E1020K}\) was constitutively active, we hypothesized that it might bypass requirements for interactions between ICOS and its ligand (ICOS-L) in the development of T_{FH} cells. To test this, we transferred naive \(\text{Pik3cd}^{E1020K/+}\) or \(\text{Pik3cd}^{E1020K/+}\) OT-II cells into wild-type mice, which we then immunized with NP-OVA in alum and treated with a blocking antibody to ICOS-L (anti-ICOS-L) (Fig. 4d). Treatment with anti-ICOS-L decreased the frequency of \(\text{Pik3cd}^{E1020K/+}\) OT-II cell markers but failed to effectively block the differentiation of \(\text{Pik3cd}^{E1020K/+}\) OT-II T_{FH} cells (Fig. 4c), despite its reducing the frequency of endogenous wild-type T_{FH} cells in the same mice (Supplementary Fig. 4e). Furthermore, although treatment with anti-ICOS-L reduced the frequency of both T_{FH} cells and GC B cells in intact wild-type mice, neither population was abolished in the mutant mice (Supplementary Fig. 4f–i). Thus, activated PI3K\(\delta\) overcame requirements for ICOS–ICOS-L interactions in the differentiation and maintenance of T_{FH} cells.

In vitro stimulation of naive OT-II cells with OVA peptide (amino acids 232–339) revealed augmented induction of activation markers on mutant cells (Supplementary Fig. 4i), suggestive of increased sensitivity to stimulation. Similarly, when we assessed phosphorylation of the kinase AKT and ribosomal protein S6 induced via the T cell antigen receptor plus the co-receptor CD28 (downstream ‘readouts’ of PI3K\(\delta\) activity), we observed enhanced phosphorylation of AKT and S6 in \(\text{Pik3cd}^{E1020K/+}\) CD4\(^+\) T cells relative to that in wild-type CD4\(^+\) T cells (Fig. 4f), as seen in patients’ cells\(^3\). Activated phosphorylated AKT (p-AKT) phosphorlates transcription factors of the FOXO family, which leads to their sequestration outside of the nucleus and subsequent degradation\(^2\). Intriguingly, in the absence of FOXO1, T_{FH} cells are also generated independently of ICOS\(^4\). To evaluate FOXO1 in this context, we activated T cells in vitro to induce expression of ICOS, allowed them to ‘rest’ and then re-stimulated them with anti-ICOS. After being activated in vitro, wild-type CD4\(^+\) T cells included a distinct population of cells that stained for p-FOXO1, and this population increased further after re-stimulation with anti-ICOS (Fig. 4g). In contrast, \(\text{Pik3cd}^{E1020K/+}\) T cells exhibited abundant p-FOXO1 even before re-stimulation with anti-ICOS (Fig. 4h). Furthermore, expression of FOXO1\(^{AAA}\), an AKT-resistant FOXO1 mutant\(^6\), decreased the frequency of mutant T_{FH} cells generated in vivo to a frequency similar to that of \(\text{Pik3cd}^{E1020K/+}\) FOXO1\(^{AAA}\) OT-II T_{FH} cells (Fig. 4b), in addition to reducing the overall accumulation of T cells, as previously reported\(^1\) (data not shown). Thus, the expression of activated PI3K\(\delta\) bypassed the need for ICOS to phosphorylate and inactivate FOXO1, an inhibitor of T_{FH} cell differentiation.

**Increased abundance of GC B and plasma cells but diminished antigen-specific responses.** Despite their greater T_{FH} cell differentiation than that of \(\text{Pik3cd}^{E1020K/+}\) OT-II cells after immunization with NP-OVA, the transfer of \(\text{Pik3cd}^{E1020K/+}\) OT-II cells into wild-type hosts was not sufficient to generate a greater number of GC B cells, at least within the time frame of analysis (Supplementary Fig. 4j). To investigate B cell–intrinsic roles of activated PI3K\(\delta\), we transferred naive \(\text{Pik3cd}^{E1020K/+}\) or \(\text{Pik3cd}^{E1020K/+}\) MD4 B cells (which have transgenic expression of a hen-egg lysozyme (HEL)-specific BCR), together with naive \(\text{Pik3cd}^{E1020K/+}\) OT-II cells, into wild-type mice, which we then immunized with a fusion of HEL and an OVA peptide of amino acids 323–339 (ref. \(^7\)) (Fig. 5a). At 8 d after immunization, \(\text{Pik3cd}^{E1020K/+}\) OT-II cells acted similarly in the presence of either \(\text{Pik3cd}^{E1020K/+}\) MD4 B cells or \(\text{Pik3cd}^{E1020K/+}\) MD4 B cells (Supplementary Fig. 5a). However, \(\text{Pik3cd}^{E1020K/+}\) MD4 B cells expanded more (Fig. 5b) and displayed greater differentiation into GC B cells (Fig. 5c) than did \(\text{Pik3cd}^{E1020K/+}\) MD4 B cells, indicative of cell-intrinsic phenotypes.

In vitro stimulation of \(\text{Pik3cd}^{E1020K/+}\) MD4 follicular B cells with HEL revealed greater induction of activation markers in those cells than in \(\text{Pik3cd}^{E1020K/+}\) MD4 B cells (Supplementary Fig. 5g), suggestive of a lower threshold of activation, despite comparable expression of activation markers before stimulation. We also observed greater proliferation of and incorporation of the thymidine analog BrdU into mutant follicular B cells in response to various stimuli in vitro (Fig. 5d,e), consistent with B cell–intrinsic effects of PI3K\(\delta\)\(^{E1020K}\). Additionally, in vitro activation of mutant B cells led to greater plasma-cell differentiation (Fig. 5f, left, and Supplementary Fig. 5h) and less switching to IgG1 (Fig. 5f, right), both of which occurred mainly in cells that had undergone extensive proliferation, as in wild-type cultures. These phenotypes paralleled findings obtained in vivo and were reversed by a selective inhibitor of PI3K\(\delta\) (Fig. 5g). Together these data indicated that PI3K\(\delta\)\(^{E1020K}\) caused B cell–intrinsic increases in the outgrowth of GC B cells and plasma cells but impaired the capacity of B cells for class-switched antigen-specific responses.

PI3K\(\delta\)\(^{E1020K}\) results in reduced death of GC B cells. In GCs, B cells undergo high rates of death, which results in the culling of low-affinity and non–antigen-specific B cells that is needed to generate proper antigen-specific antibody responses\(^8\). In contrast, ex vivo \(\text{Pik3cd}^{E1020K/+}\) GC B cell populations included a greater frequency of live GC B cells and less staining for activated caspase-3 than that of their wild-type counterparts (Fig. 5h,i). That enhanced survival seemed to be B cell intrinsic, as mixed–bone marrow chimeras generated with wild-type and mutant cells had a greater frequency of
Fig. 4 | Pik3cd<sup>−/−</sup> T cells intrinsically generate more T<sub>fh</sub> cells in ICOS-dependent manner. a, Experimental protocol for b,c: wild-type (CD45.1<sup>+</sup>) host mice were given transfer of Pik3cd<sup>−/−</sup> OT-II (CD45.2<sup>+</sup>) cells (n = 7 hosts) or Pik3cd<sup>−/−</sup> E1020K/+-OT-II (CD45.2<sup>+</sup>) cells (n = 6 hosts) 1 d before (d-1) intraperitoneal (i.p.) immunization with NP-OVA in alum (on day 0), followed by analysis of the spleen 8 d after (+8). b, Frequency of CD45.2<sup>+</sup> OT-II cells among live cells from mice as in a (donor in key). c, Flow-cytometry analysis of the expression of PD-1 and CXCR5 by OT-II (donor) cells from mice as in a (donor, above plots) (left), and frequency of PD-1<sup><sub>+</sub></sub>CXCR5<sub>+</sub> OT-II T<sub>fh</sub> cells (key in b) (right). Numbers in outlined areas (left) indicate percent PD-1<sup><sub>+</sub></sub>CXCR5<sub>+</sub> cells. d, Experimental protocol for in vivo treatment with anti-ICOS-L: host mice given cell transfer and immunization as in a also received isotype-matched control antibody (Isotype) or anti-ICOS-L intravenously (i.v.) on day -1 and intraperitoneally on days +1, +3 and +5 and were analyzed on day +7 (host mice: n = 5 (Pik3cd<sup>−/−</sup> OT-II donor cells) or n = 4 (Pik3cd<sup>−/−</sup> E1020K/+-OT-II donor cells) for isotype-matched control antibody; n = 4 (Pik3cd<sup>−/−</sup> E1020K/+-OT-II donor cells) or n = 5 (Pik3cd<sup>−/−</sup> E1020K/+-OT-II donor cells) for anti-ICOS-L)). e, Flow cytometry as in c of cells from mice as in d (donors above plots) (left), and frequency of PD-1<sup><sub>+</sub></sub>CXCR5<sub>+</sub> OT-II T<sub>fh</sub> cells (right). f, Flow-cytometry analysis of AKT phosphorylated at Ser473 (p-AKT<sup>Ser473</sup>) and of S6 phosphorylated at Ser235 and Ser236 (p-S6<sup>Ser235/236</sup>) in wild-type or Pik3cd<sup>−/−</sup> naive CD4<sup>+</sup> T cells given pretreatment with an inhibitor of PI3K<sub>x</sub> (+CAL-101) or vehicle (key) and left unstimulated (Ex vivo) or stimulated for 30 min with anti-CD3 and anti-CD28 (above plots); numbers in plots indicate gMFI (colors match key). g, Flow-cytometry analysis of FOXO1 phosphorylated at Ser273 (p-FOXO1<sup>Ser273</sup>) in wild-type or Pik3cd<sup>−/−</sup> naive CD4<sup>+</sup> T cells activated in vitro for 4 d, allowed to ‘rest’ for 1 h in RPMI medium, then left unstimulated (US) or stimulated with anti-ICOS (key) (left), and frequency of p-FOXO1<sup>T</sup> cells (same key) (right). h, Flow-cytometry analysis of the expression of PD-1 and CXCR5 by OT-II cells isolated from wild-type host mice (n values below) given transfer of Pik3cd<sup>−/−</sup> OT-II cells (n = 5) or Pik3cd<sup>−/−</sup> E1020K/+-OT-II cells (n = 4) transduced to express green fluorescent protein (GFP) alone (gated on GFP<sup>+</sup> cells), or Pik3cd<sup>−/−</sup> E1020K/+-FOXO1<sup>AAA</sup> OT-II cells (n = 5) or Pik3cd<sup>−/−</sup> E1020K/+-FOXO1<sup>AAA</sup> OT-II (n = 3) cells transduced to express GFP and FOXO1<sup>AAA</sup> tagged with hemagglutinin (HA) (gated on GFP<sup>+</sup>HA<sup>+</sup> cells) (above plots), followed by intraperitoneal immunization with NP-OVA and analysis 5 d after immunization (left), and frequency of OT-II PD-1<sup>+</sub>CXCR5<sub>+</sub> T<sub>fh</sub> cells (right). Numbers adjacent to outlined areas (left) as in c. Each symbol (b,c,e,h) represents an individual mouse. Data are representative of three (a-c,g) or two (d-f,h) independent experiments, with cells pooled from two to four mice per group in f,g (mean ± s.e.m. in b,c,e,h). *P < 0.05 and **P < 0.01 (Mann-Whitney U-test).
GC B cells, as well as live cells within the mutant GC B cell compartment (Supplementary Fig. 5i)). Although differences in the in vitro steady-state survival of unstimulated naïve follicular B cells were not observed (Supplementary Fig. 5k), various stimuli, including anti-IgM and lipopolysaccharide (LPS), increased the frequency of live mutant proliferating cells (Supplementary Fig. 5l). Moreover, IL-4 increased the survival of mutant B cells relative to that of wild-type B cells, even in the absence of proliferation (Supplementary Fig. 5m). In contrast, BAFF did not increase survival of mutant B cells but consistently led to the proliferation of mutant B cells, a result not observed for wild-type cells (Supplementary Fig. 5n), which directly linked activated PI3Kδ to those phenotypes. Thus, PI3Kδδ1020E/ increased both the survival and the proliferation of activated B cells.

FOXO proteins transcriptionally activate genes encoding pro-apoptotic molecules, such as Bel2L1, which encodes the BCL-2 family member BIM. As in T cells, we observed increased phosphorylation of AKT and S6 (Fig. 5j), as well as a greater frequency of p-FOXO1+ Pik3cδδ1020E/ B cells than of wild-type cells (Fig. 5k), after in vitro stimulation. Consistent with those observations, mutant GC B cells exhibited lower expression of BCL-2 family member MCL-1 for proteasome degradation28. Accordingly, β inhibits the kinase GSK3, which directly linked activated PI3Kδδ1020E/ to the kinase GSK3δ, that of wild-type cells (Fig. 5l). AKT-mediated phosphorylation also increased the expression of MCL-1 (Fig. 5m), which encodes the BCL-2 family member MCL-1 for proteasome degradation29. Accordingly, in vitro stimulation resulted in higher expression of MCL-1 in mutant B cells than in wild-type B cells (Fig. 5n). Thus, activated PI3Kδδ1020E/ regulated multiple pathways that promote B cell survival.

Pik3cδδ1020E/ mice display elevated serum autoantibodies. The induction of B cell apoptosis within the GC is essential for the maintenance of self-tolerance through the elimination of self-reactive clones30. Notably, many patients with APDS are positive for autoantibodies and develop autoimmune manifestations, such as glomerulonephritis. Similarly, we found a higher concentration of IgG and IgM anti-nuclear antibodies in the serum of mutant mice than in that of wild-type mice (Fig. 6a and Supplementary Fig. 6a). Evaluation with an array of 94 different autoantigens revealed that IgM and IgG antibodies directed against approximately 50 autoantigens had significantly greater reactivity in mutant mice than in wild-type mice (Fig. 6b, Supplementary Fig. 6b and Supplementary Table 2). Moreover, 1-year-old mutant mice exhibited lymphocytic infiltration in multiple organs (Fig. 6c). Thus, Pik3cδδ1020E/ mice exhibited increased self-reactive antibodies and lymphocytic infiltration of tissues that worsened with age.

Increased gut-associated GCs with a greater abundance of IgA-coated fecal bacteria. It is now appreciated that there are numerous connections between the commensal microbiome and autoimmunity31. To evaluate whether the microbiota contributed to the activated phenotypes of Pik3cδδ1020E/ mice, we assessed mesenteric lymph nodes (mLNs) and Peyer’s patches (PPs), which are continuously exposed to a wide range of microbiota and food-derived antigens that sustain the generation of Tfh cells and GC B cells32. Again, we found increased cellularity, as well as a greater number of Tfh cells and GC B cells, in mutant mLNs and PPs than in their wild-type counterparts (Fig. 7a,b). Furthermore, whereas wild-type mLNs had GCs predominantly in the cortical region, mutant mLNs displayed a greater abundance of GCs scattered throughout the internal medullary area (Supplementary Fig. 7a,b). Mutant mLNs also displayed disorganization of GCs, with poor demarcation of DZ and LZ areas, and infiltration of PD-1+ Tfh cells into the DZ (Supplementary Fig. 7a,c,d), as in peripheral LNs.

Tfh cells have a crucial role in supporting the selection of GC B cells and their differentiation into IgA-secreting plasma cells in response to commensal stimulation in gut33. Accordingly, a greater abundance of free fecal IgA, as well as a greater frequency of fecal IgA-coated bacteria, were detected in Pik3cδδ1020E/ mice than in wild-type mice.
To identify the taxa of gut bacteria 'preferentially' targeted by IgA, we sorted IgA-coated and uncoated bacteria and sequenced 16S rRNA in wild-type and Pik3cd<sup>E1020K/+</sup> mice. Many taxa of bacteria exhibited greater IgA coating in Pik3cd<sup>E1020K/+</sup> mice than in wild-type mice. Although these results did not reach statistical significance after correction for multiple testing, these taxa outnumbered those less targeted by IgA in Pik3cd<sup>E1020K/+</sup> mice than in wild-type mice (Fig. 7e and Supplementary Table 3).

Intriguingly, these included Akkermansia muciniphila, a commensal bacterium that is very abundant in the human gut<sup>32</sup> (Fig. 7f). The overall community structure of the microbiota of wild-type mice did not differ consistently from that of mutant mice (Supplementary Fig. 7e,f). However, phylogenetic diversity, which reflects the richness in number and phylogenetic distribution of taxa within a single community, was significantly lower in mutant mice and was inversely correlated with the frequency of IgA-coated bacteria.
mice (Fig. 8a), indicative of greater systemic responses to gut commensal bacteria in the serum of mutant mice than in that of wild-type mice. We detected a significantly higher frequency of IgG2a/b that bound specifically to fecal bacteria. Mutant serum antibodies that bound A. muciniphila, but not to the control bacterium Lactobacillus reuteri, in mutant mice than in wild-type mice (Fig. 8b), in support of the proposed specificity of these responses.

Furthermore, mutant splenic follicular B cells showed greater responsiveness to autologous fecal microbiome extracts and a lower threshold for the expression of activation markers in response to autologous fecal microbiome extracts (Fig. 8c), as well as to LPS, an innate TLR agonist released by gut bacteria (Fig. 8d), relative to that of wild-type splenic follicular B cells. Thus, the Pik3cd<sup>E1020K/+</sup> mutation increased both antigen-specific reactivity and innate reactivity to commensal-derived products.

**Antibiotics diminish activated phenotypes and autoantibodies.** To evaluate potential connections between anti-commensal responses and autoimmunity, we assessed the antibodies that specifically bound fecal bacteria. Mutant serum antibodies that bound...
Fig. 7 | Altered homeostasis of gut-associated lymphoid tissue, with increased IgA-coated fecal bacteria in Pik3cd<sup>E1020K/+</sup> mice. a, Cellularity of mLN and PPs in 10-week-old naive wild-type and Pik3cd<sup>E1020K/+</sup> (WT) mice (key). b, Quantification of GC B cells (B220<sup>+</sup>CD19<sup>+</sup>GL-7<sup>+</sup>FAS<sup>+</sup>) and T<sub>FH</sub> cells (CD4<sup>+</sup>B220<sup>−</sup>PD-1<sup>−</sup>CXCR5<sup>+</sup>Foxp3<sup>+</sup>) in the mLN and PPs of mice as in a (n = 5 wild-type and n = 6 Pik3cd<sup>E1020K/+</sup>). c, ELISA of IgA in fecal washes of mice as in a (n = 3 per group) at various dilutions (horizontal axis), presented as the optical density at 405 nm (OD<sub>405</sub>) (key as in a). d, Flow-cytometry analysis of feces from mice as in a (above plots) with the red fluorescent nucleic-acid stain Syto-62 (Syto; for the detection of bacterial DNA) and with anti-IgA (left), and the frequency of IgA-coated bacteria in such mice (right). Numbers adjacent to outlined areas (left) indicate percent SYTO<sup>+</sup> (bacteria-containing) feces (left plot of each) or fecal IgA-coated bacteria (right plot of each). e, IgA scores of coated bacteria in Pik3cd<sup>E1020K/+</sup> mice relative to those in wild-type mice (mean fold values), for various families (symbol color; top key), plotted against P values (symbol size; bottom key). f, Bacterial taxa (below plot) with greatest difference in IgA-coating scores in wild-type mice relative to those in Pik3cd<sup>E1020K/+</sup> mice, assessed by serial Mann-Whitney U-tests (above plot). On all taxa detected in at least four mice per group, followed by calculation of Benjamini-Hochberg q values (above plot, top), FDR, false-discovery rate. g, Faith’s phylogenetic diversity in wild-type and Pik3cd<sup>E1020K/+</sup> mice (key). h, Correlation between alpha diversity (as in g) and overall frequency of IgA-coated fecal bacteria in Pik3cd<sup>E1020K/+</sup> (n = 6). P = 0.0167 (Spearman). Each symbol (a,b,d,g) represents an individual mouse. Data are representative of three independent experiments (a-c,e-h) with n = 8 wild-type mice and n = 9 Pik3cd<sup>E1020K/+</sup> mice (e,f) or n = 8 wild-type mice and n = 6 Pik3cd<sup>E1020K/+</sup> mice (g) (mean ± s.e.m. in a-c,g, median and interquartile ranges in f) or are pooled from five independent experiments with n = 8 wild-type mice and n = 21 Pik3cd<sup>E1020K/+</sup> mice (d, mean ± s.e.m.). * P < 0.05, ** P < 0.01 and *** P < 0.001 (Mann-Whitney U-test (a,b,d) or unpaired t-test (g)).

gut bacteria and were subsequently eluted showed greater binding of double-stranded DNA than that of their wild-type counterparts (Fig. 8c), suggestive of possible cross-reactivity with self antigens.

To determine whether gut commensals directly contributed to the immune-system activation in Pik3cd<sup>E1020K/+</sup> mice, we treated wild-type and mutant littersmates with a ‘cocktail’ of antibiotics for 6 weeks, beginning at the time of weaning. Treatment with antibiotics reduced splenic cellularity, as well as the number of activated CD4<sup>+</sup> T cells and T<sub>FH</sub> cells and the frequency and number plasma cells and plasmablasts, in the spleen of mutant mice, but not in that of wild-type mice (Fig. 8d–i). Notably, treatment of mutant mice with antibiotics reduced the abundance of total IgG, as well as antibodies directed against double-stranded DNA and various self antigens, to levels seen in untreated wild-type mice (Fig. 8j–l). Thus, the generation of hyperactivated and autoreactive phenotypes in Pik3cd<sup>E1020K/+</sup> mice required signals derived from commensal stimuli.

**Discussion**

In a mouse model that recapitulated features of APDS, we found a greater abundance of T<sub>FH</sub> cells and GC B cells but inefficient class-switched antigen-specific B cell responses to immunization. Nonetheless, the Pik3cd<sup>E1020K/+</sup> mice exhibited a greater abundance
of antibodies to commensal bacteria and self antigens, the latter of which were strikingly diminished by treatment with antibiotics. Our data suggested that Pik3cd selectively controlled lymphocyte activation, allowing the efficient generation of protective responses while preventing excessive reactivity to tonic stimulation by commensals that can promote lymphoid dysregulation.

Several factors might have contributed to the aberrant humoral immunity of Pik3cd<sup>E1020K</sup>/+ mice. T<sub>Fr</sub>, cells, which are normally confined to the LZ, specifically target high-affinity B cells, inducing survival and proliferation. In contrast, an increased number of T<sub>Fr</sub> cells might diminish competition for the survival signals provided to B cells and paradoxically impair specific responses<sup>32</sup>. Furthermore, T<sub>Fr</sub> cells abnormally located in the DZ in mutant GCs might drive inappropriate signals<sup>31</sup>, including PI3K-mediated pathways in B cells. Of note, patients with APDS also show disrupted GC architecture with increased invasion by T<sub>Fr</sub> cells<sup>14</sup>. This mis-localization of T<sub>Fr</sub> cells is intriguing, given that PI3K pathways regulate multiple molecules involved in lymphocyte migration<sup>1</sup>. Our analyses also revealed an increased abundance of Foxp3<sup>+</sup> regulatory T cells and T<sub>Fr</sub> cells in mutant mice. However, while an increased ratio of T<sub>Fr</sub> cells to T<sub>Fr</sub> cells can impair humoral responses<sup>14</sup>, we observed a ratio in mutant mice similar to that of wild-type mice. Finally, an increased abundance of GC B cells and T<sub>Fr</sub> cells, which completely fill the FDC networks over time, probably contribute to the impaired development of new antigen-specific responses, particularly with age.

Like FOXO1-deficient T cells<sup>1</sup>, mutant CD4<sup>+</sup> T cells exhibited ICOS-independent differentiation into T<sub>Fr</sub> cells. However, despite published data indicating a more important role for Pik3cd in T<sub>Fr</sub> cells than in GC B cells<sup>1</sup>, we found that Pik3cd<sup>E1020K</sup>/+ B cells intrinsically generated more GC B cells and plasma cells, with increased p-FOXO1. Mirroring the requirements for FOXO1 in DZ formation, regulation of the cytidine deaminase AID and the generation of antigen-specific responses<sup>30,31</sup>, Pik3cd<sup>E1020K</sup>/+ mice also had defective switching to IgG1 and a lower ratio of DZ to LZ. Thus, the phenotypes of cells in Pik3cd<sup>E1020K</sup>/+ mice recapitulated the phenotypes of FOXO1-deficient T cells and B cells, although they were less severe.

Published data obtained with an activated, ubiquitously expressed p110α isoform expressing the proposal that p110α has a role in tonic BCR signaling required for B cell survival<sup>32</sup>. Similarly, we found increased survival of Pik3cd<sup>E1020K</sup>/+ activated B cells, associated with altered expression of BIM and MCL-1. Interestingly, BIM-deficient mice succumb to systemic lupus erythematosus–like autoimmunity with accumulation of self-reactive lymphocytes and autoantibodies<sup>18</sup>. MCL-1 is critical for the formation of memory B cells and plasma cells<sup>7,18</sup>. Together these factors might impair the selective pruning required for proper antigen-specific responses.

Pik3cd<sup>E1020K</sup>/+ mice exhibited greater lymphoid activation associated with gut-associated lymphoid tissue and a greater frequency of both fecal IgA–binding fecal bacteria and serum IgG–binding fecal bacteria. There is enrichment for IgA-coated bacteria among commensal pathogens that can drive intestinal and systemic inflammation in particular disease settings<sup>41</sup>. Notably, we detected GCs in the medullary region of mutant mLN<sub>s</sub>, a finding that is normally observed only with inflammation<sup>41</sup>. Among the bacterial species ‘preferentially’ bound by IgA in mutant mice, we found <i>A. muciniphila</i>, which promotes IgA responses<sup>21,34</sup>, and is associated with protective effects in diabetic models<sup>43</sup> and cancer immunotherapy<sup>44</sup>. We speculate that alterations in the prevalence of substantially IgA-coated bacteria might amplify autoreactive responses under certain genetic conditions, such as in Pik3cd<sup>E1020K</sup>/+ mice.

Our data support the proposal of a strong connection between the microbiota and autoimmunity in APDS. Systemic responses to gut bacteria could neutralize commensals that spread systemically when gut barriers are leaky<sup>19,27</sup>, as has been proposed for people deficient in the adaptor MYD88 or the kinase IRAK-4, who have a greater abundance of B cells that express the heavy-chain variable region <i>V<sub>H</sub></i>4–34, which cross-reacts with self antigens and commensals<sup>28</sup>. However, immune responses to microbiota might also amplify autoreactive lymphocytes, causing immunopathology in peripheral sites, as reported for other autoimmune conditions<sup>32</sup>. While altered gut integrity might contribute to increased anti-commensal reactivity, mucosal barriers seemed to be grossly intact in the mutant mice. We therefore speculate that activated Pik3cd lowers the threshold for signaling from receptors on T cells and B cells, making them more susceptible to activation by commensal products physiologically released in the periphery. Although we found IgG reactive to <i>A. muciniphila</i>, but not IgG reactive to the control bacteria <i>Lactobacillus</i> or to phospholipid, which is used to identify polyreactive antibodies (data not shown), we cannot rule out the possibility of a general relaxation of tolerance and non-antigen-specific activation by gut bacteria. However, the elevated abundance of commensal antigen-specific IgG, in conjunction with the increased responsiveness of mutant B cells to autologous fecal material and LPS, would suggest that activated Pik3cd drives hyper-responsiveness to both antigens and innate stimuli. In contrast, these commensal stimuli might be suboptimal for the triggering of downstream pathways in wild-type cells with appropriately controlled Pik3cd activity.

Fig. 8 | Greater commensal reactivity and diminished activated phenotypes after antibiotic treatment in Pik3cd<sup>E1020K</sup>/+ mice. a. Flow-cytometry analysis of feces of wild-type and Pik3cd<sup>E1020K</sup>/+ mice incubated with autologous serum and stained with Syto-62 (as in Fig. 7d) and with anti-IgG2a/b (detects binding of IgG2c/b in C57BL/6 mice) (left), and frequency of serum IgG2a/b-coated bacteria (right). Numbers adjacent to outlined areas (left) indicate percent Syto<sup>+</sup> (bacteria-containing) feces (left plot of each) or IgG2a/b-coated fecal bacteria (right plot of each). b. MFI of <i>A. muciniphila</i> (left) or <i>L. reuteri</i> (right) bound by serum IgG in wild-type and Pik3cd<sup>E1020K</sup>/+ mice (key). c,d,gMFI of CD86 on wild-type or Pik3cd<sup>E1020K</sup>/+ splenic naive follicular B cells stimulated for 20 h in vitro with autologous fecal bacterial extracts, in the presence (+) or absence of treatment with an inhibitor of Pik3cd (key) (c), or stimulated for 20 h in vitro with various concentrations (horizontal axis) of LPS (d). e, ELISA of IgG directed against double-stranded DNA (dsDNA IgG) in serum from wild-type and Pik3cd<sup>E1020K</sup>/+ mice and in serum antibodies (Abs) stripped from commensals obtained as in a (key). f. Spleen cellularity in wild-type and Pik3cd<sup>E1020K</sup>/+ littersmates treated for 6 weeks, beginning at the time of weaning, with antibiotics plus a sweetener (+ Abx) or with sweetener alone (key in k). g,h. Quantification of splenic CD4<sup>+</sup> CD4<sup>+</sup> T cells (g) and splenic T<sub>Fr</sub> cells, (CD4<sup>+</sup>B220<sup>-</sup>PD-1<sup>-</sup>CXCR5<sup>-</sup>Foxp3<sup>-</sup>) (h) in mice as in f (key in k). i, Flow cytometry of live cells from mice as in f (above plots) (left), and quantification of splenic plasma cells and plasmablasts (CD138<sup>+</sup>B220<sup>-</sup>) in mice as in f (key in k) (right). j,k. ELISA of IgG (j) and of IgG to double-stranded DNA (k) in serum of mice as in f (key in k). l, Array analysis of IgG autoantibodies (self antigens; one per row) in serum from mice as in f and MRL–NZM mice (above plot), presented as in Fig. 6b (key). Each symbol (a,b,e-k) represents an individual mouse. Data are pooled from five independent experiments with n=19 wild-type mice and n=21 Pik3cd<sup>E1020K</sup>/+ mice (a; mean + s.e.m.); are from one experiment with n=8 wild-type mice and n=7 Pik3cd<sup>E1020K</sup>/+ mice housed in three to four independent cages per genotype (b; mean + s.e.m.); are representative of two independent experiments with a pool of three mice per group (c,d); are representative of three independent experiments with n=7 Pik3cd<sup>E1020K</sup>/+ mice and n=6 Pik3cd<sup>E1020K</sup>/+ mice (e; n=5 wild-type mice per group and n=4 Pik3cd<sup>E1020K</sup>/+ mice per group (f–h,j,k) or n=4 mice per group (i) (e–k; mean + s.e.m.); or are from one experiment with n=3 mice per genotype (no antibiotics) or n=4 mice per genotype (antibiotics), or n=2 MRL–NZM mice (l). *P<0.05, **P<0.01 and ***P<0.001 (Mann-Whitney U-test).

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Our findings emphasize the importance of PI3Kδ in the activation of T cells and B cells required for proper GC reactions, in which both too little PI3Kδ activity and too much PI3Kδ activity disrupt humoral immunity. Notably, we have identified a previously unappreciated role for PI3Kδ in modulating responses to the commensal microbiota that, when not properly controlled, led to lymphoid hyper-reactivity that worsened with age. Published work has shown promising results for the treatment of patients with APDS with an inhibitor of PI3Kδ; however, the long-term consequences of this treatment remain unknown, especially given data demonstrating genomic instability associated with blockade of PI3Kδ. Our work might provide new perspectives on managing patients with activated PI3Kδ, as well as other autoimmune conditions, including those induced by checkpoint-blockade therapy, in which dampening PI3K pathways might allow the selective control of immune responses.
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Author contributions
S. Preite designed and performed experiments, analyzed and interpreted data and wrote the manuscript; J.C. performed manipulations and injections of embryonic stem cells; S.V. and K.D. analyzed autoantibody arrays; B.H. helped with experiments and provided discussions; A.I. performed, designed and performed experiments and provided discussions; S.V. and K.D. performed and analyzed immunohistochemistry and confocal microscopy experiments; I.-C.V. designed, performed and analyzed gut and microbiome experiments; J.-G.R. designed, performed and analyzed ‘activated PI3Kδ’ mouse model and provided discussions; S.V. and K.D. analyzed autoantibody arrays; B.H. helped with experiments and provided discussions; J.C. performed manipulations and injections of embryonic stem cells; and R.O. provided technical support; I.R. and C.Z. helped generate and analyze autoantibody array data; Q.-Z.L. designed the array and analyzed the data; M.O.L. provided Rosa26-HA-hFoxo1 mice.
mice. S. Pittaluga provided experimental advice; G.U. provided patient samples and discussions; L.D.N., Y.B. and R.N.G. provided reagents and intellectual input; and P.L.S. conceived of the project, wrote the manuscript and provided overall direction for the study.

**Competing interests**
The authors declare no competing interests.

**Additional information**
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Methods

Patient samples. All human subjects and their guardians in this study signed written informed consent in accordance with Helsinki principles for enrollment in research protocols that were approved by the Institutional Review Board of NIAID (clinical trials registration number NCT00001355, US NIH). Blood donors were obtained at the NIH Clinical Center under approved protocols. All procedures were based on standard of care, under established clinical guidelines. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque gradient centrifugation. Blood T<sub>eff</sub> cells were stained at 20°C (room temperature) using antibodies indicated in the Nature Research Reporting Summary and figure legends.

Mice. C57BL/6 mice (000664), OT-II mice (004194), ZP3-Cre mice (003651), CD45.1<sup>+</sup> (002014), BLIMP-1-YFP mice (008828) and MD4 mice (002359) were obtained from The Jackson Laboratory. Rosa26-h-HumanTG (Foxo1<sup>AAA</sup>) mice, were bred to OT-II or CD45.2<sup>+</sup> mice. Mice were genotyped for the targeted allele were crossed to ZP3-Cre mice to delete the allele. Sorted and were injected intravenously into wild-type hosts that were immunized intraperitoneally with NP-OVA, 48 h later, followed by analysis on day +5 in the spleen.

Antigens and immunization. For OT-II cell-transfer experiments, mice were immunized intraperitoneally with 50 μg of NP-OVA (BioXcell Technologies) in alum (Imject Alum Adjuvant, Thermo Scientific). For co-transfer of MD4 cells and OT-II cells, mice were immunized intraperitoneally with 25 μg of a fusion of wild-type HEL and OVA peptide (amino acids 323–339) in alum (provided by Humabs BioMed). For subcutaneous immunization, mice received 20 μg of NP-OVA in alun in the hock. Analyses were performed 7–8 d after immunization.

In vitro antibody blockade of ICOS-L. For OT-II cell-transfer experiments, 100 μg of anti-ICOS-L (H.K5.3, Bioxell) or isotype-matched control antibody (2A3, BioXcell) was given intravenously on day –1 and then intraperitoneally every other day until the day the mice were killed. For the treatment of wild-type and Pik3cd<sup>E1020K/+</sup> mice, antibodies (identified above) were given intravenously on day 0 and intraperitoneally on days +2, +4, +6.

In vitro experiments. For in vitro B cell proliferation, live naive follicular B cells were isolated using anti-CD3-conjugated microbeads, then were sorted as B220<sup>+</sup>CD19<sup>+</sup>CD23<sup>+</sup>CD21<sup>+</sup>GL<sup>-</sup> FAS<sup>D</sup> D38<sup>+</sup>Anti-Aqua/Live/Dead<sup>+</sup> on a FACSARIA (BD Biosciences). CD21<sup>+</sup>/CD23<sup>+</sup> cells were enriched with a T cell negative-selection kit (Miltenyi Biotec), then CD4<sup>+</sup> and CD8<sup>+</sup> dendritic cells (DCs) were enriched using a naive CD4<sup>+</sup> T cell isolation kit and anti-CD43- conjugated microbeads (Miltenyi Biotech). In some experiments, T cells (CD4<sup>+</sup>CD8<sup>+</sup> T cells) were isolated using a naive CD4<sup>+</sup> T cell isolation kit and anti-CD43-conjugated microbeads (Miltenyi Biotech). In some cultures, T cells and B cells were co-cultured for 4 d, then were re-stimulated for 4 d with the appropriate antigen and cultured for an additional 4 d.

Histology. Mouse organs were fixed overnight at 20°C (room temperature) in neutral buffered formalin. After fixation, samples were dehydrated in 70% ethanol and embedded in paraffin, cut on a microtome (8–10 μm) and stained with hematoxylin and eosin (Histoserv). Immunohistochemical images were acquired on an upright Zeiss Axios Imager D2 microscope (Carl Zeiss) with a 1 x., 5 x or 10 x objective lens, using an AxiosCam HRc full color CCD camera. Zeiss ZEN blue pro 2011 software package was used for collection and for post-processing of the images.
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flow cytometry–sorted wild-type or mutant OT-II non-T<sub>n</sub> cells (PD-1<sup>-</sup> CXCXR5<sup>-</sup>) and T<sub>n</sub> cells (PD-1<sup>-</sup> CXCXR5<sup>+</sup>) isolated after transfer into wild-type mice 7 d after immunization. OT-II cells were re-stimulated in vitro for 4 h with the phorbol ester PMA (Sigma, 20 ng/ml), ionomycin (1 μg/ml Sigma) and GolgStop (1 μg/ml, BD Biosciences) and were stained for IL-21 using recombinant mouse IL-21R-Fc (CD43<sup>-</sup>) isolated from naive wild-type spleen, together with polyclonal wild-type co-cultures (T cell/B cell ratio, 1:1) were performed using wild-type B cells (CD43<sup>+</sup>) isolated from naive wild-type spleen, together with polyclonal wild-type or mutant flow cytometry–sorted T<sub>n</sub> cells (CD4<sup>-</sup>CD25<sup>-</sup> PD-1<sup>-</sup>CXCXR5<sup>+</sup>), isolated on day +7 from the spleen of mice immunized intraperitoneally with NP-OVA, and were re-stimulated for 7 d with anti-IGM (5 μg/ml) and anti-CD3 (1 μg/ml). Microbiome extracts were prepared from stool samples isolated from the large intestine of wild-type and mutant mice as previously described<sup>5</sup> Complete Mini Protease Inhibitor Cocktail (Roche) and 0.5 mM phenylmethylsulfonyl fluoride (Sigma) were used to prepare the protease inhibitor ‘cocktail’ for re-suspension of the bacterial pellet. Protein extract concentration was calculated with a Pierce BCA Protein Assay Kit. Flow cytometry–sorted naive splenic wild-type or mutant follicular B cells were stimulated for 20 h in vitro with serial dilutions of the extract and were treated with 1 μg/ml of anti-CD10 or not. All antibody information is provided in the Nature Research Reporting Summary.

Immunohistochemistry and confocal microscopy. Immunohistochemistry was performed as previously described<sup>15</sup>. Organs were harvested and then fixed for 3 h at 4°C. Cryo/Premount (Electron Microscopy Services) was used to freeze the tissue and the tissue was then cryostat-sectioned (1 μm), washed in PBS and were incubated in 30% sucrose overnight before being embedded in OCT compound (Tissue-Tek). 30-μm sections were cut on a CM3050S cryostat (Leica) and were adhered to Super Frost Plus Gold slides (Electron Microscopy Services). Frozen sections were permeabilized and were blocked for 1–2 h in PBS containing 0.3% Triton X-100, 1% BSA and 1% Fc block. Sections were re-blocked with 10% FCS for 2 h at 4°C in a humidity chamber in the dark. All antibody information is provided in the Nature Research Reporting Summary. Cell nuclei were visualized with JO-1 (Thermo Fisher Scientific). Stained slides were mounted with Fluoromount G (Biosciences) and were sealed with a glass coverslip. Digital images were captured using a Nikon Eclipse 50i microscope with a ×2.5 objective (NA 0.14), 2 HyD and 3 PMT detectors, and six lasers (UV, argon, dpSS, OMe, HeNe, HeNe and diode) capable of nine excitation wavelengths (405, 458, 476, 488, 514, 561, 594, 633 and 690 nm). All images were captured at 8-bit depth, with a line average of 3, and 1,024 × 1,024 format with the following pixel dimensions: x (0.284–0.378 μm), y (0.284–0.378 μm), and z (1–1.25 μm).

Histocytometry. Histocytometry analysis was performed as previously described<sup>28</sup>. An eight-color panel was developed consisting of the following fluorophores: Brilliant Violet 421, Brilliant Violet 510, Alexa Fluor 488, JOJO-1, PE, Alexa Fluor 488, Brilliant Violet 510, DZ (CD35<sup>lo</sup> and BCL-6<sup>hi</sup>). These positional gates were then applied to PD-1<sup>+</sup> surfaces to create TFH cells (PD-1<sup>-</sup>CXCR5<sup>-</sup>) for subcellular expression levels of the GC area, TFH cell surfaces were first created in Imaris using expression levels of DZ (CD35<sup>lo</sup> and BCL-6<sup>hi</sup>). These numbers were then divided by the area of each region at a voxel density of 1,024 × 1,024 and 1-μm<sup>3</sup> extraction followed by ethanol precipitation and purification using the QIAquick QIAZOL (Thermo Fisher Scientific). Stained slides were mounted with Fluoromount G (Biosciences) and were sealed for 24 h with BD CytoFix/CytoPerm (BD Biosciences) diluted in PBS<sub>1</sub> hant parasite of the nuclear genome, and the parasite was not detected in either the IgA<sup>-</sup> or IgM<sup>-</sup> samples. IgA scores were generated from each sample, and a position–gene expression value was calculated as the ratio of total reads sequenced to the total number of reads sequenced. IgA scores were calculated for each region within each sample mouse pair (IgA<sup>-</sup> and IgA<sup>+</sup>) by log2 transformation of the ratio of the average abundance of the non-IgA-coated fraction from the abundance of the IgA-coated fraction. Taxis that were not detected in either the IgA<sup>-</sup> fraction or the IgA<sup>+</sup> fraction for a given mouse were excluded from statistical analyses on a per-mouse basis.

In vivo antibiotic treatment. Wild-type and Pkx3<sup>cld<sub>a</sub></sup><sup>−/−</sup> littermates were separated at weaning and were placed in different cages on the basis of their genotype. Unfractionated bacterial pellets, prepared as described in the previous section, were stored at –80°C until use. In parallel, IgA-coated fecal bacteria were stained with anti-IgA-PE (Nature Research Reporting Summary) followed by anti-PE-conjugated microbeads (Miltenyi Biotec). Samples were enriched for IgA<sup>+</sup> and IgA<sup>-</sup> bacteria by passing through the antibody resuspended at a density of 1×10<sup>9</sup> colony-forming units per staining condition. Bacterial pellets were incubated in heat-killed serum diluted to 1:50 and washed and were subsequently stained with the following, alone or in combination: anti-IGG (AB5; BD Biosciences); anti-IgG<sub>2a</sub> (X57; BD Biosciences), which cross-reacts with IgG<sub>2b</sub> in the BS to determine the number of IgG-coated bacteria (previously incubated with serum as described above) were pelleted and washed four times with SB to remove the unbound fraction; bound antibodies were stripped from the pellet using 0.1 M glycine, pH 2.9, and for incubation for 30 s, and were spun for 2 min at maximum speed in a microcentrifuge. Supernatant containing IgG was recovered and put in 1/10 volume of Tris, pH 8, and was assessed by ELISA for binding to dsDNA.

Gut microbiota sequencing. Wild-type and Pkx3<sup>cld<sub>a</sub></sup><sup>−/−</sup> littermates were separated at weaning and placed in different cages on the basis of their genotype. Unfractionated bacterial pellets, prepared as described in the previous section, were stored at –80°C until use. In parallel, IgA-coated fecal bacteria were stained with anti-IgA-PE (Nature Research Reporting Summary) followed by anti-PE-conjugated microbeads (Miltenyi Biotec). Samples were enriched for IgA<sup>+</sup> and IgA<sup>-</sup> bacteria by passing through the antibody resuspended at a density of 1×10<sup>9</sup> colony-forming units per staining condition. Bacterial pellets were incubated in heat-killed serum diluted to 1:50 and washed and were subsequently stained with the following, alone or in combination: anti-IGG (AB5; BD Biosciences); anti-IgG<sub>2a</sub> (X57; BD Biosciences), which cross-reacts with IgG<sub>2b</sub> in the BS to determine the number of IgG-coated bacteria (previously incubated with serum as described above) were pelleted and washed four times with SB to remove the unbound fraction; bound antibodies were stripped from the pellet using 0.1 M glycine, pH 2.9, and for incubation for 30 s, and were spun for 2 min at maximum speed in a microcentrifuge. Supernatant containing IgG was recovered and put in 1/10 volume of Tris, pH 8, and was assessed by ELISA for binding to dsDNA.
RNA was isolated using a Qiagen microRNA isolation kit and was transcribed into cDNA. Taqman probes were used with TaqMan Universal PCR Master Mix (Thermo Scientific) according to the manufacturer’s instructions to amplify Bcl2l11 mRNA (Mm00437796_m1). 18S (4319413e, Thermo Scientific) was used to normalize and calculate relative expression.

Statistical analysis. Data were analyzed via Prism 6 (GraphPad Software) or custom R scripts and the ‘Hmisc’ package, using parametric t-tests and the non-parametric unpaired Mann-Whitney U-test for comparison of two unpaired groups. The mean and s.e.m. are presented in the figures. Significance is indicated in the figures as follows: *P < 0.05, **P < 0.01 and ***P < 0.001. If not indicated, the P values were not significant (> 0.05). PERMANOVA was performed using the ‘adonis’ function in the R package ‘vegan’.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The materials, data, and any associated protocols that support the findings of this study are available from the corresponding authors upon request. Accession codes for microbiome sequences are publicly available at NCBI (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP132959). The custom IgA-seq analysis R scripts that support the findings of this study are available upon request from Ivan Vujkovic-Cvijin (ivc@nih.gov).

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- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Software and code

Policy information about availability of computer code

Data collection

- Softmax Pro 5.4.5, LASX 1.6 software, Zeiss ZEN blue pro 2011, BD FACSDIVA™ SOFTWARE.

Data analysis

- Softmax Pro 5.4.5, Bitplane Imaris, ImageJ 1.50i, Photoshop, Flowjo9.9, Flowio v10.1r5, Genepix Pro 6.0 softwares, Multi experiment viewer software (MeV, DFCI Boston, MA), Prism 6 (GraphPad Software), exactRankTests’ R package and the ‘Hmisc’ package.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No statistical method was used to predetermine sample size. Sample size was determined based on previous and/pilot studies. In most experiments between 4-6 mice were used per group and guided by the 3R principles to reduce animal numbers.

Data exclusions

No data were excluded from the analysis.

Replication

Biological and technical replicates have been performed. Most of the experiments have been reproduced 2-3 independent times with comparable results. Information on replicates is included in figure legends. Where representative data were shown, the experimental findings were reproduced with similar results.

Randomization

For human samples: PI3Kd patients have been screened genetically prior to this work. Healthy donors (>18y age) were received from NIH blood bank, no information on these individuals is available.

For mouse experiments: littermates from multiple breeding were randomly allocated to experiments but based on genotype, age and sex to control for covariates.

Blinding

For most of the experiments, investigators were not blinded. However, in most mouse experiments, isolation of organs and cells from mice were performed in a blinded fashion by a third party. The genotypes of patients and mice were known during the data collection and/or analysis because we chose objective readouts as measure of our experiments. Therefore, the outcome and results were not prone to be influenced by subjective evaluation. Sup. Fig8b was blinded during data collection and analysis. Quantifications of cells in Figure 3g and Sup. Fig. 7a were performed by software independently of human factor.

Reporting for specific materials, systems and methods

Materials & experimental systems

Methods

n/a Involved in the study

n/a Involved in the study

Antibodies

Antibodies used

Antibody, clone, vendor, cat.#, lot#, dilution:

MOUSE ANTIBODIES:
CD3-BV421, 145-2C11, BioLegend, 100335, B237072, 1:200
CD4-BV605, RM4-5, BioLegend, 100548, B244080, 1:500
CD8-APC-Cy7, 53-6.7, eBioscience, 47-0081-82, 4322567, 1:200
CD19-APC-Cy7, 1D3, BioLegend, 115530, B253924, 1:100
CD19-PerCP/Cy5.5, 1D3, BioLegend, 152406, B241294, 1:100
CD25-PE, PC61, BioLegend, 102008, B191542, 1:300
CD25-FITC, PC61, BioLegend, 102006, B152996, 1:100
CD62L-PE/Dazzle 594, MEL-14, BioLegend, 104448, B219195, 1:400
CD62L-AF647, MEL-14, BioLegend, 104421, B239264, 1:400
CD44-AF700, IM7, eBioscience, 56-0441-82, 4329936, 1:100
CD45.1-PE/Dazzle 594, A20, BioLegend, 110747, B211844, 1:300
CD45.1-BV421, A20, BioLegend, 110732, B211844, 1:300
CD45.2-FITC, 104, eBioscience, 11-0454-85, E00316-1632, 1:300
CD86-AF488, GL-1, BioLegend, 105017, B178153, 1:100
PD-1-PE-Cy7, RMP1-30, BioLegend, 109110, B218703, 1:200
ICOS-APC-Cy7, C398.4A, BioLegend, 313530, B234257, 1:100
B220-PerCP, RA3-6B2, BioLegend, 103234, B232968, 1:100
B220-APC-eFluor780, RA3-6B2, eBioscience, 47-0452-82, 4317799, 1:100
FAS-PE-Cy7, Jo2, BD Biosciences, 557653, 6214949, 1:200
FAS-BV421, Jo2, BD Biosciences, 562633, 7047594, 1:200
GL-7-APC, BioLegend, 144606, B207559, 1:400
GL-7-Pacific Blue, BioLegend, 144613, B209951, 1:200
CXCR5-biotin, 2G8, BD Biosciences, 551960, 7068617, 1:50
CXCR5-purified, 2G8, BD Biosciences, 551961, 6343832, 1:100
CXCR4-biotin, 12G5, BD Biosciences, 551968, 6028693, 1:200
CD21-FITC, 7E9, eBioscience, 11-0212-82, 4293549, 1:200
CD23-PE, B3B4, eBioscience, 12-0232-82, E01138-226, 1:200
CD138-BV605, 281-2, BioLegend, 142516, B232065, 1:500
IgM-FITC, II/41, eBioscience, 11-5790-81, 4282654, 1:100
IgM-PE-Cy7, R6-60.2, BD Biosciences, 552867, 6083739, 1:100
IgD-AF647, 11-26c-2a, BioLegend, 405707, B247514, 1:300
IgD-PE, 11-26c-2a, BioLegend, 405705, B241543, 1:300
IgG1-BB515, X56, BD Biosciences, 565104, 7128657, 1:200
CD43-FITC, S7, BD Biosciences, 553270, 2317604, 1:200
Foxp3-eFluor450, M89-61, BD Biosciences, 562465, 1:50
p-AKT S473-PETexas red, M89-61, BD Biosciences, 562465, 1:50
p-S6 S240/244 AF647, D68F8, Cell Signaling Technology, 50448, 1:500
p-S6 S235/236 PE-Cy7, D57.2.2E, Cell Signaling Technology, 344115, 1:500
CD93-BV421, AA4.1, eBioscience, 48-5892-80, E17714-102, 1:500
CD5-APC, 53-7.3, eBioscience, 17-0051-82, 4318517, 1:200
CD69-PE-Cy7, H1.2 F3, BioLegend, 104512, B253212, 1:200
HA-AF647, 6E2, Cell Signaling Technology, 344854, 1:500
Recombinant Mouse IL-21 R Fc Chimera, 596-MR, R&D, 1:20
R-Phycocerythrin AffiniPure F(ab')2 Fragment Anti-Human IgG, Fcy Fragment Specific, 109-116-098, Jackson Laboratories, 1:50
SECONDARY ANTIBODIES
AF488 goat anti-rabbit IgG (H+L), Invitrogen, A11034, 1885241, 1:500
Biotin-SP (long spacer) AffiniPure Fab Fragment Goat Anti-Rat IgG (H+L), Jackson ImmunoResearch, 112-067-003, 1:1000
Brilliant Violet 421™ Streptavidin, BioLegend, 405226, 1:500
Streptavidin-APC, BD Biosciences, 554067, 1:500

Validation
All antibodies used in this study are from commercial sources and have been validated by the vendors. Validation data are available on the manufacturer's website. Appropriate antibody dilutions were performed based on preliminary experiments and intensity of fluorescent signals. Dilutions for flow cytometry antibodies are referred to a staining volume of 20-30ul per sample (1-3 x 10^6 cells).

Eukaryotic cell lines
Policy information about cell lines

Cell line source(s)
HEK 293 T cells were obtained from ATCC.

Authentication
No method for cell authentication was used.

Mycoplasma contamination
No mycoplasma contamination test was performed because: cells are kept in culture for no more than 12 passages and new lots were obtained from ATCC every 6-12 months.

Commonly misidentified lines
Used cell lines are not listed in the ICLAC database.

Animals and other organisms
Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Male and female Mus musculus mice have been used. C57BL/6 (000664), OT- II (004194), ZP3-Cre (003651) MD4 (002595) and BLIMP-1-YFP (008828), mice were obtained from The Jackson Laboratory. Pik3cdE1020K/+ mice have been generated as described in the method section.
In most of the experiments mice have been analyzed at 8 or 16 weeks of age. In certain experiments, 12-14 month old mice were analyzed. Age information is provided in each figure legend. Mice were maintained and treated under specific pathogen-free (SPF) conditions in accordance with the guidelines of the NHGRI Animal Care and Use Committee at the National Institutes of Health under protocol number NHGRI G98-3.

Wild animals
n/a

Field-collected samples
n/a

Human research participants
Policy information about studies involving human research participants

Population characteristics
PBMC samples from seven PI3Kd patients (PASLI/APDS) have been used in this study, with the following characteristic: age, sex, ethnicity, point mutation in p110d, treatment (TRX):
25 years, M, CAUC, E525K, NO TRX
5.5 years, F, CAUC, E1021K, NO TRX
15 years, F, AA, N334K, SIROLIMUS
25 years, F, CAUC, E1021K, NO TRX
16 years, F CAUC, E525K, NO TRX
11 years, M, CAUC, E1021K, EVORILIMUS
29 years, F, ASIAN, E1021K, NO TRX
Blood from healthy donors, age 18 or older, was obtained at the NIH Clinical Center under approved protocols.

Recruitment
PASLI/APDS patient samples were selected based on genetic screening for mutation is PI3Kd. No information was obtained regarding these patients based on NIH blood bank policy.
### Flow Cytometry

#### Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

#### Methodology

**Sample preparation**

For human samples: Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque gradient centrifugation. For FACS staining: cells were washed and resuspended in MACS buffer (PBS with 2%FBS and 2μM EDTA), block with 10% human serum in PBS, washed twice and stained in MACS buffer with antibody mix.

For mouse samples: Spleens and peripheral lymph nodes were minced between the 3ml syringe plunger and fine mesh in MACS buffer (PBS with 2%FBS and 2μM EDTA) to obtain single-cell suspensions that were then filtered through a 70 micron filter. After ACK (Ammonium Chloride) lysis of RBCs, cells were washed once and Fc receptors blocked with anti-mouse CD16/32 (2.4G2, Bio X Cell). Cells (1-3 × 10^6 cells) were incubated with antibodies for 45/60 min on ice.

Mouse and human samples have been stained in round bottom 96 well plate in 20-30ul of antibody mix.

**Instrument**

- LSRII (BD Biosciences).
- FACSaria Illu cell sorter (BD Biosciences).

**Software**

- BD FACSDIVA™ was used to collect flow cytometry data.
- FlowJo 9.9 software (TreeStar) was used to analyze data.

**Cell population abundance**

- Post-sort fractions had more than 95% purity that was verified through flow cytometry analysis on the same FACSaria machine used to sort the cells.

**Gating strategy**

- Initial gating strategy performed: FSC-A/SSC-A, exclusion of doublets (through SSC-H/SSC-W and FSC-H/FSC-W), live cells (negative for Aqua or Near-IR dead stain kit). These were followed by specific gating strategy reported in the figure legends of main and supplementary figures.

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.