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The ubiquitin ligase Itch skews light zone selection in germinal centers

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Abstract

Immunoglobulin (Ig) diversification occurs in peripheral lymphoid organs, after establishment of central tolerance during B cell development. In germinal centers (GC), somatic hypermutation of Ig genes occurs in Dark Zones (DZ), followed by selection of mutated clones in Light Zones (LZ). This generates high affinity Ig receptors to pathogens, but can also produce autoreactive Ig receptors, which are removed by selection mechanisms that are incompletely understood. The ubiquitin ligase Itch prevents the emergence of autoimmune disease and autoantibodies in humans and mice, and patients lacking Itch develop potentially fatal autoimmune diseases; yet how Itch regulates GC B cells is not well understood. By studying Itch deficient mice, we have recently shown that Itch directly limits the magnitude of GC responses. Proteomic profiling of GC B cells uncovered that Itch deficient cells exhibit high mTORC1 and Myc activity, hallmarks of positive selection. Bone marrow chimera and adoptive transfer experiments revealed that B cell Itch restricts non-cycling LZ cells. These results support a novel role for Itch in skewing selection of GC B cells to restrict LZ accumulation and shape GC-derived humoral immunity. Determining how B cells integrate cues within GCs to navigate through LZs and DZs will aid in understanding how autoreactive clones emerge from GCs in those suffering from autoimmune disease.

Introduction

Germinal centers (GCs) are transient microanatomic sites in which activated B cells undergo affinity maturation prior to differentiating into long-lived plasma cells or memory cells (1). Upon entering GCs, B cells undergo proliferation and somatic hypermutation (SHM) of immunoglobulin (Ig) genes followed by selection, in which some cells are culled. GC B cells proliferate every 5–12 hours (1, 2), but about 50% of GC B cells undergo apoptosis in the same time frame (3). As such, there is massive cell turnover with relatively stable total numbers of GC cells.

Proliferation/SHM and selection are physically separated within GCs. In light zones (LZ), GC B cells gather antigen through Ig receptor binding and endocytosis. Presentation of peptide-MHCII to cognate follicular helper T cells (Tfh) positively selects approximately 10% of cells to enter cell cycle and move to the dark zone (DZ), and the rest die by neglect (3, 4). DZ cells proliferate to an extent proportional to the amount of antigen that was

presented (5, 6)(5, 24, 4). Cells that re-express a functional Ig receptor return to the LZ for another round of selection (2, 7). The affinity and quantity of GC-derived antibodies are determined by decisions of individual B cells to live, die, proliferate, or exit the GC in response to T cell help and antigen. GC selection decisions modify the central tolerance-defined Ig receptor repertoire, and GCs can produce autoreactive clones from tolerant cells or redeem autoreactive anergic cells (8–10)

The mechanisms underlying GC B cell decisions are only beginning to be understood. The LZ is the site where B cells integrate selection cues from antigen and T cell help and either return to the DZ, die, or differentiate and exit to the plasma cell or memory pool. In this context, CD40 signaling, triggered by CD40 ligand (CD40L) on cognate T follicular helper (Tfh) cells activates Myc and mTORC1, which initiate cell growth and determine the numbers of divisions that will occur in the DZ (11–13). Accordingly, elevated Myc levels and mTORC1 activity during positive selection lead to expansion of DZ cells. T cell help appears to dictate the extent of proliferation of DZ GC B cells, but Ig receptor signaling also contributes to GC selection. In GC B cells, CD40 ligation alone cannot activate mTORC1, and both Ig receptor and CD40 is required. Ig receptor signaling can also increase GC cell numbers and the proportion of LZ GCs (14, 15).

The above mechanisms have in part elucidated the external cues that drive GC selection, but the regulation of internal pathways that connect these cues to B cell responses are less well understood. Nuclear localization of the transcription factor Foxo1 promotes DZ proliferation, but Syk-dependent Ig receptor signaling and PI3 kinase signaling reduce nuclear levels of Foxo1, increasing the percentage of LZ cells (15–18)(20, 26). Cbl ubiquitin ligases degrade the transcription factor IRF4 to promote cyclic reentry to the DZ and limit premature exit of low affinity plasma cells (19). The ubiquitin ligase March1 promotes turnover of peptide-MHCII molecules in DZ cells, such that cells returning to the LZ must recapture antigen at each GC round (20). Over multiple rounds of selection, subtle GC B cell-intrinsic defects in signal transduction, antigen presentation, cell cycle, or apoptosis could result in substantial changes in antibody levels, affinity, and specificity (e.g. autoreactivity).

The ubiquitin ligase Itch is required to prevent humoral autoimmunity in humans and mice (21–27). Itch is a member of the Nedd-4 family of catalytic E3 ubiquitin ligases (28). As such, Itch directly binds to its substrates and catalyzes the covalent attachment of ubiquitin molecules to the substrates, targeting them for degradation or changing their subcellular location. Itch deficiency in humans is rare, but can cause severe autoimmunity, characterized by high levels of autoantibodies and a variety of organ-specific autoimmune diseases, in some cases with multiple diseases presenting in a single patient(21–23, 29). In mice, Itch deficiency results in spontaneous inflammation at mucosal sites, expansion of activated T and B cells, and formation of autoantibody(27, 30–33). Itch has been well studied in T cells, where it ubiquitylates transcription factors to tune T helper cell differentiation into Th2, Th17, Treg, and Tfh cells(27, 31, 34, 35). Less is known about how Itch regulates antibody formation, but recent studies have identified that Itch directly limits antigen-driven humoral responses (36, 37) and has a subtle role in B cell development(38).

Our lab has previously shown that within B cells, Itch limited proliferation and mTORC1 activity and reduced the total numbers of spontaneous and antigen-driven GC B cells. In mixed chimeras, Itch KO GC B cells outcompeted WT GC B cells, suggesting that Itch changed the way GC B cells responded to selection and survival cues (36). Here, we show that Itch limits not only the magnitude of antigen-driven GC responses, but also the skewing of LZ and DZ frequencies. We show that Itch deficient mice have both larger and more numerous GCs, but are less reliant on Tfh cell help. Itch directly limits hallmarks of LZ selection and the frequency of LZ GC B cells in Itch KO mice, mixed chimeras, and in an adoptive transfer model. Mechanistically, Itch deficiency enhanced the accumulation of LZ, but not DZ, GC cells in the G1 phase of cell cycle. Together, these results are consistent with the idea that Itch limits LZ B cell proliferation to enforce the requirement for Tfh cell help in antigen-driven humoral responses.

Materials and Methods

Mice

Itch KO mice (aka Itchy mice (32, 33)) and B1-8 mice (39) were crossed to generate Itch KO B1-8 mice. For all experiments using B1-8 transgenic mice, mice were heterozygous for the B1-8 heavy chain. For experiments using WT and Itch KO mice, B1-8 transgene negative littermates were used. CD45.1 mice (JAX stock #002014) were crossed to WT B1-8 mice to generate WT B1-8 CD45.1/2 heterozygous mice for mixed BM chimeras. Mice were bred and housed first at the Children's Hospital of Philadelphia, then moved to the University of Florida. All breeding, housing, and procedures followed IACUC-approved protocols.

Generation of bone marrow chimeras

For mixed chimeras, CD45.1 recipient mice were lethally irradiated (1100 RAD) then injected via the tail vein with 2 million bone marrow cells, 50% from WT CD45.1/2 B1-8 and 50% Itch KO CD45.2 B1-8 mice. Prior to injection, activated lymphocytes were depleted from bone marrow cells using anti-CD5 magnetic beads. Mice were kept on antibiotic water for 3 weeks after irradiation. The transplanted immune system was allowed to mature for 8 weeks prior to immunization or other analysis.

Immunization

Mice were immunized by intraperitoneal injection with 200ug NP-OVA with a conjugation ratio of 16NP/OVAL (cat. #N-5051; Biosearch Technologies) adsorbed to alum. NP-ova/alum was made in house as follows: A 0.5 mg/ml solution of NP-OVA was mixed with 10% aluminum potassium sulfate, and the pH was adjusted to 6.5. After overnight incubation, the precipitate was collected by centrifugation, washed with PBS, then resuspended in PBS to give a final concentration of 0.25 mg/ml.

CD40 blockade

Mice were injected with 2× 200ug of a-CD40L (Bio X Cell cat. #BE0017-1) or isotype (Bio X Cell cat. #BE0091) by intraperitoneal injection. Doses were given on day 0 and day 2, then mice were harvested on day 3.

Proteomics

Flow cytometry-sorted WT and Itch KO GC B cells from B1-8 mixed chimeras were pelleted and frozen at −80°C prior to proteomics analysis. Proteomics analysis was carried out at the Children's Hospital of Philadelphia Proteomics Core. Samples were first subject to in-solution digestion: Cell pellets were lysed, solubilized, and digested with the iST kit (PreOmics GmbH, Martinsried, Germany) per manufacturers protocol. The resulting peptides were de-salted, dried by vacuum centrifugation and reconstituted in 0.1% TFA containing iRT peptides (Biognosys Schlieren, Switzerland). Samples were then subject to mass spectrometry acquisition and analysis: Peptides were analyzed on a QExactive HF mass spectrometer (Thermofisher Scientific San Jose, CA) coupled with an Ultimate 3000 nano UPLC system and an EasySpray source using data independent acquisition (DIA). Raw MS data was searched using the direct-DIA mode in Spectronaut software and downstream analyses were performed in Perseus(40–43).

Flow Cytometry and Antibodies

Flow cytometry analysis was carried out on either a 5-laser Cytek Aurora (at the Children's Hospital of Philadelphia flow cytometry core) or a 3-laser Cytek Aurora (at the University of Florida Center for Immunology and Transplantation flow cytometry core). Live/dead discrimination was done using Live/dead blue (Invitrogen # L23105) or Zombie aqua (BioLegend cat. #423102). All antibody staining was carried out in the presence of Fc Block (BioLegend cat. #101320). All antibodies were from BioLegend unless otherwise noted. For LZ/DZ GC staining: CD19 (cat, #115543), IgM (cat. #406512), IgD (cat. #405712), CD38 (cat. #102742), GL7 (eBiosciences cat. #53590282), CXCR4 (cat. #146511), CD86 (cat. #105030). For Tfh staining: CD3 (cat. #100204), CD4 (cat. #100547), CXCR5 (cat. #145510), PD-1 (cat. #135205). CXCR5-biotin antibodies were incubated for 1 hour at 37 degrees C prior to surface staining with remaining surface antibodies plus streptavidin-A647 (cat. #405237). Other surface antigens: CD45.1 (Clone A20), CD45.2 (Clone 104). NP-binding B cells were detected with NP-PE (Biosearch Technologies cat. Cat. #N-5070-1). BrdU and 7AAD were stained per the manufacturer's instructions (BD 552598). All flow cytometry analysis was carried out using FlowJo10 (BD). Cell Sorting was carried out on a MoFlo Astrios Sorter (BD) at the Children's Hospital of Philadelphia flow cytometry core.

Immunohistochemistry

Spleens from WT and Itch KO mice were formalin fixed, sliced lengthwise, and submitted to the Molecular Pathology Core at the University of Florida. Spleens were embedded in paraffin and sectioned, then stained for PNA (Vector Labs cat #B-1075-5). From each spleen, 3 sections >200um apart were cut. In brief, after citrate retrieval, 1:200 dilution of anti-PNA was incubated on the slides for 1 hour. After primary, the DAB labeling was performed (Vector Laboratories). Stained slides were scanned, and GC numbers and area were quantified using Aperio Image Scope (v12.4.3.5008).

Statistics

Statistical methods are stated in each figure legend, and statistics were calculated using Prism7 for Mac OS X software (GraphPad). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

Results

Unimmunized Itch deficient mice have larger and more numerous GCs

We previously showed that Itch KO mice have increased numbers of GC B cells in the spleen, as defined by cell surface markers. GCs are microanatomical structures that can be visualized in histological sections of secondary lymphoid tissues using peanut agglutinin (PNA). Increased GC B cells could occur due to increased GC number or size. We collected spleens from 9–10 week old WT and Itch KO mice, and we enumerated PNA+ structures within follicles in histologic sections (Figure 1A). We found that Itch KO mice had more numerous GCs in spleen cross-sections (Figure 1B), which could indicate that spontaneous GCs form more often or persist longer in a setting of Itch deficiency. They also have larger GCs (Figure 1C–D), consistent with the idea that GC B cells expand more or die less. Spontaneous GCs are relatively rare in spleens of WT mice. Peyer's patches are sites of constant exposure to microbial antigens, where GCs are expanded in normal mice as a part of active tolerogenic mucosal immunity, especially primed to induce IgA production (44, 45). We examined GCs in Peyer's patches from WT and Itch KO mice by flow cytometry. We found that proportions and numbers of GC B cells was comparable in Peyer's patches from WT and Itch KO mice (Supplemental Figure 1). These data support the idea that Itch prevents spontaneous GC activity in the spleen, associated with autoimmune diseases, and we focused our studies on GCs in the spleen.

Itch deficient GC B cells are sustained by fewer Tfh

GC B cells require frequent interactions with specialized Tfh cells. GC B cells present MHCII-antigen to Tfh, and in turn receive help in the form of cytokines and costimulation through surface receptors (e.g. CD40-CD40L)(46, 47). GC B cell and Tfh cell numbers are highly interdependent, and cognate interactions increase the numbers of both cell types in an antigen dose-dependent manner (48). A previous report has demonstrated that Itch deficient Th cells are unable to differentiate into Tfh in the context of viral infection(34). This result seemed at odds with the large numbers of spontaneous GCs in Itch KO mice. We identified spontaneous Tfh cells (Figure 2A) and GC B cells (Figure 2B) in the spleens from Itch KO mice using flow cytometry. As we have shown before, Itch KO mice have dramatically increased numbers of GC B cells, but the numbers of Tfh are only slightly elevated (Figure 2C). We quantified the ratios of spleen GC B cells per Tfh within each mouse. In WT mice, one Tfh supports approximately 1.5 GC B cells, but in Itch KO mice, one Tfh supports approximately 4 GC B cells (Figure 2D). These data show that more Itch deficient GC B cells can be supported by a limited number of Tfh.

Itch deficient GC B cells could have enhanced responses to Tfh costimulation, which could explain why there was a higher GC to Tfh ratio in Itch KO mice. To disrupt the key receptor-ligand interactions between GC and Tfh, we injected WT and Itch KO mice with

anti-CD40L. After 3 days, we enumerated the spontaneous GC and Tfh in the spleen. We found that blocking CD40L dramatically reduced the numbers of GC B cells in WT mice, but only slightly decreased the numbers of GC cells in Itch KO mice, and this was not statistically significant (Figure 2E). Numbers of Tfh were not significantly decreased after 3 days in either WT or KO mice (Figure 2F). In individual mice, the GC:Tfh ratio remained significantly higher in Itch KO mice compared to WT mice, even with blockade of CD40-CD40L interactions (Figure 2G). This is consistent with the idea that Itch deficient B cells are less dependent on CD40 signaling for survival.

Itch KO GC cells show evidence of increased Myc, mTORC1, and oxidative phosphorylation

Lack of Itch led to increased numbers of GC B cells that persisted even with defective Tfh cell help. In order to determine how Itch limited GC B cells, we screened the whole proteomes of WT and Itch deficient GC B cells. We wanted to set up a system in which we could generate large numbers of WT and Itch KO GC B cells with equal access to T cell help. We set up mixed chimeras by transferring bone marrow from WT B1-8 and Itch KO B1-8 mice into a lethally irradiated host. After the transplanted immune system was allowed to mature for 8 weeks, the mice were immunized with NP-ova in alum. After 2 weeks, WT and Itch KO spleen GC B cells were sorted by flow cytometry (Figure 3A). Digested lysates from WT and Itch KO GC B cell pellets from 4 chimeras were analyzed by liquid chromatography-tandem mass spectrometry. Approximately 1500 proteins were identified in each sample (Figure 3B), and there was nearly 100% overlap in the identity of the proteins found in WT and Itch KO samples, with only 3 proteins found in Itch KO B cells only (Ighm, Wapl, and G3bp2) (Figure 3C). The protein levels were compared in paired samples from individual mice, and 227 proteins were found to be statistically significantly up- or down- regulated in Itch deficient GC B cells (Figure 3D). We queried these 277 proteins using the Broad Institute's Molecular Signatures Database Hallmarks gene set (49). The top 3 most statistically significant Hallmarks indicated were Myc Targets, mTORC1 Signaling, and Oxidative Phosphorylation (Figure 3E). Both Myc and mTORC1 transiently increase in GC B cells that have been positively selected (11, 13). GC B cells rely primarily on oxidative phosphorylation, not glycolysis, to maintain rapid proliferation (50). For the proteins within these Hallmarks, we examined the protein levels within our proteomics dataset. Almost all the proteins in each Hallmark showed higher levels in the Itch KO GC B cells (Figure 3F). Based on this screen, we would expect that Itch deficient GC B cells would have an advantage in LZ selection and proliferation.

Itch deficiency increases proportions of LZ GC cells in the presence of antigen

Itch deficient GC B cells in mixed chimeras (with equal access to Tfh cell help) showed an increased Myc and mTORC1 signature, associated with positive selection in GC LZs. We wanted to determine whether Itch had an impact on the LZ and DZ distribution of GC B cells. We examined LZ and DZ GC B cells from WT and Itch KO mice using flow cytometry. We found that proportions of LZ cells in Itch KO GCs were increased approximately 30% over WT GCs, with a corresponding decrease in Itch KO DZ GC B cells (Figure 4A). Itch KO mice develop inflammatory lesions in the skin, lung, and gut, due to the activity of unrestrained Th2 cells. The expansion of GC LZ in Itch KO mice

could be due to the inflammatory environment, which would favor high levels of damage and inflammation-induced self-antigens. We again examined mixed chimeras using the same strategy as in Figure 3A. As we have previously shown(36), Itch KO GC B cells make up a disproportionately large fraction of the GC in mixed chimeras, and Itch KO cells made up the majority of spontaneous GCs, total GCs after immunization, and NP+ GC B cells after immunization (Supplemental Figure 2A–B). We examined the LZ and DZ GC B cells in these mice before and after immunization. Prior to antigen exposure, there was no skewing of LZ and DZ B cell frequencies (Figure 4B). After immunization with NP-ova/alum, Itch deficient GC B cells showed a skewing toward increased percentages of LZ cells (Figure 4C). This indicated that WT and KO B cells responded differently to equal levels of antigen and Tfh cell help, and this suggests that a competitive advantage may favor increased proportions of LZ cells. The requirement for antigen in driving LZ skewing in the mixed chimeras was somewhat surprising, given that increased LZ cells were observed in spontaneous GCs from unimmunized Itch KO mice. This may be due to the fact that Itch KO mice experience increased antigen exposure compared to WT mice due to systemic inflammatory disease (33), such that both antigen availability and increased B cell responsiveness contributed to expanded LZs in Itch KO mice. In mixed chimeras, both WT and KO GC B cells experienced equal access to such antigens of inflammation. The additional strong stimulus of the T-dependent antigen, NP-ova, was needed to demonstrate increased responsiveness of Itch KO B cells, resulting in increased LZ proportions.

Itch limits accumulation of G1 cells in the LZ

How could LZ cells take over a greater proportion of the GC? An increased proportion of LZ cells could be caused by changes in proliferation or survival within each compartment, or cycling between compartments. To examine the role of Itch in GC B cell proliferation and survival, we set up an adoptive transfer system using WT and Itch KO B1-8 transgenic cells. We transferred 20,000 WT or Itch KO lambda+ cells into WT congenic recipients, and immunized the mice with NP-ova in alum. We examined the LZ and DZ GC B cells on day 12, when we have previously seen increased numbers of Itch KO GC cells in this model (Figure 5A). We quantified numbers of splenic LZ and DZ B1-8 GC B cells, and we found that the numbers of both LZ and DZ Itch KO GC cells were increased compared to WT (Figure 5B). Despite the increase in both compartments, the proportions of LZ cells were approximately 20% higher in Itch KO B1-8 cells compared to WT B1-8 cells (Figure 5C). This indicated that Itch deficiency caused an accumulation of GC B cells in the LZ at a higher rate than the DZ.

We next wanted to examine whether there were any differences in cell death or proliferation that might explain this skewing in LZ and DZ proportions. Using the same adoptive transfer system, we examined apoptosis in LZ and DZ GC B cells by staining for activated Caspase 3/7 in B1-8 GC cells on day 12 after transfer. As expected, we found that apoptotic DZ cells were twice as frequent as apoptotic LZ cells. However, Itch deficiency did not reduce cell death in either compartment (Figure 5D–E). We next examined proliferation by analyzing BrdU uptake. On day 12 post adoptive transfer and immunization, we injected 1mg BrdU, intraperitoneally. After 1 hour, mice were sacrificed, and spleen B cells were stained for LZ/DZ GC markers, BrdU incorporation, and DNA content. We found that approximately

10–15% of GC cells both DZ and LZ cells incorporated BrdU one hour after injection. We were surprised that there was not increased BrdU incorporation in DZ compared to LZ cells, since DZ cells have been reported to have increased proportions of cycling cells in polyclonal and B1-8 germinal centers at day 7–8 post immunization (4). We examined BrdU incorporation in the non-transgenic recipient polyclonal and B1-8 transgenic LZ and DZ GC B cells at day 12 post immunization, paired between individual mice. We found that there was a slight (1.3-fold) increase in the percent of BrdU+ DZ cells compared to LZ cells in the polyclonal GC B cells (Supplemental Figure 3A), but there was no change in the B1-8 cells (Supplemental Figure 3B). In a subset of experiments, we stained NP-binding GC B cells with fluorescently labeled NP. Among the $CD45.2^+$ B1-8 cells, more than 70% of GC B cells were NP binders, and approximately 30% of the CD45.1 host GC cells were NP binders (data not shown). Within the NP+ GC B cells, both host polyclonal (Supplemental Figure 3C) and B1-8 transgenic GC B cells (Supplemental Figure 3D) showed ~1.3-fold increased BrdU incorporation in the DZ over LZ. These data indicate that NP-binding B1-8 cells appropriately reflect the DZ/LZ proliferation dichotomy, although this is blunted in the total B1-8 population at this time point, compared to host polyclonal GC cells. Importantly, there was no difference in BrdU uptake between WT and Itch KO B1-8 cells in either LZ or DZ, or within the NP-binding subset (Figure 5F–G, Supplemental Figure 3E). This showed that there was no gross difference in the numbers of cells undergoing apoptosis or actively replicating their DNA that could be detected by examining the overall LZ and DZ compartments.

LZ and DZ GCs are heterogeneous cell populations, and cells with LZ or DZ surface markers may be in different stages of their trajectory between LZ selection and DZ proliferation and SHM. We reasoned that a subset of LZ or DZ GC cells must have increased proliferation or decreased death to account for the changes in GC cell numbers and LZ frequencies observed in Figure 5B–C. We measured DNA content to approximate the cell cycle stage of LZ and DZ cells in the adoptive transfer model. We gated on the transferred B1-8 GC population and examined BrdU vs. DNA content (measured by 7AAD). Using these parameters, we divided the population into 3 subsets, G1 ($BrdU^-7AAD^{low}$), S ($BrdU^+7AAD^{intermediate}$), and G2/M ($BrdU^-7AAD^{hi}$) (Figure 6A). We verified that there were very few host memory B cells that showed BrdU incorporation or high 7AAD staining, as an internal control (Supplemental Figure 4A). Within the cell cycle gates, we quantified the proportions of LZ and DZ cells to determine if LZ skewing was associated with a particular cell cycle stage (Supplemental Figure 4B). **Increased** proportions (~1.3-fold) of Itch KO LZ cells were only found within the G1 gate (Figure 6B), and the proportions of DZ cells in G1 were correspondingly decreased (Figure 6C). These data indicate that Itch deficiency increased the proportion of cells in the LZ by allowing the accumulation of G1 cells. G1 LZ cells are likely to be cells that are awaiting T cell help to propel them into DZs for rapid proliferation or awaiting GC exit and memory or plasma cell differentiation. Accumulation of G1 LZ cells in Itch deficiency supports the idea that Itch promotes death of LZ cells that do not quickly obtain Tfh cell help and re-enter cell cycle.

Discussion

In this report, we have shown that Itch deficiency promotes larger and more numerous GCs that are less reliant on Tfh cell help. Despite the decreased dependence on CD40L-CD40 T cell interactions, Itch KO GC B cells showed increased hallmarks of positive selection after NP-ova immunization, normally associated with T cell help. Itch limited the proportion of LZ GC B cells, and prevented accumulation of G1 LZ cells. Together, these data show that Itch regulates the way LZ GC B cells respond to T-dependent antigens, enforcing the requirement for CD40L-dependent Tfh interactions.

We have demonstrated that Itch regulates the skewing of LZ and DZ GC cells. Due to the complex nature of the GC B cell program, there are many factors that could change the LZ/DZ proportions. According to the current model of antigen-dependent selection in GCs, B cells enter GCs after receiving antigen receptor and cognate T cell help. They then undergo proliferation while inducing high levels of somatic mutations in immunoglobulin genes within DZs. Approximately 50% of the mutated DZ GC cells die, and the other 50% migrate to the LZ and test the new antigen receptor by capturing antigen from follicular dendritic cells and presenting it to Tfh cells. Successful elicitation of Tfh cell help drives about 10% of the cells back to the DZ for more proliferation and mutation (4). The quantity of cognate T cell help dictates the number of DZ divisions(13). In Itch deficiency, both LZ and DZ cell numbers are increased (~2-fold), but LZ percentages are disproportionately elevated (~1.3-fold). In the context of the GC selection model, increased LZ percentages would most likely be due to amplified LZ return, survival, or proliferation, rather than decreased proliferation in DZs. In studies using Dec 205-mediated antigen delivery to synchronize T cell help, the proportion of LZ cells expanded over the first 24 hours of antigen delivery, showing that in normal GCs, T cell help contributes to LZ expansion (13). In B cells lacking Itch, a slightly larger LZ population may reflect increased responses to T cell dependent selection signals.

Our results also indicate that Itch deficient cells can “do more with less,” and the GC/Tfh ratio is more than 4-fold higher in Itch KO than WT GCs. Furthermore, CD40L blockade does not dissolve GCs as effectively in Itch KO compared to WT mice. This could indicate that Itch KO GC B cells rely more on alternative T cell costimulation, such as Icos-IcosL (51), or Itch KO GC B cells could utilize selection signals that are not T cell dependent, including Ig receptor signaling and integrin signaling(52). In instances of suboptimal T cell help, antigen receptor signaling promotes GC B cell selection (14), and augmenting antigen receptor signals can increase the LZ proportion (15). Further studies are needed to identify the internal or external cues that drive increased LZ percentages in Itch KO B cells.

We found that Itch KO LZ cells accumulated in G1. In the GC, where the default fate is cell death, increased cell accumulation likely reflects enhanced positive selection or defective negative selection. This could indicate amplified DZ to LZ return, or that some LZ cells spend longer in G1 prior to entering S phase, or that more cells complete cell cycle in the LZ. About 50% of DZ cells return to the LZ on any given GC cycle (4). Two recent studies have determined that DZ to LZ return requires that the GC B cells express functional immunoglobulin genes (3, 7), but how the cells sense this is unknown. Itch could regulate

the DZ to LZ selection process, resulting in an increased LZ percentage and augmented numbers of cells that have recently left cell cycle. Alternatively, Itch may restrict survival of LZ cells that do not quickly obtain Tfh cell help. This would lead to accumulation of G1 LZ cells that are awaiting an opportunity to interact with cognate helper T cells. Finally, although LZ cells enter cell cycle at a similar pace to DZ cells, they are less likely to complete cell cycle in the LZ (2). Future studies that interrogate whether these accumulating LZ cells are on their way in or out of the GC would be beneficial. One approach would be to examine somatic mutations in LZ GC cells. High mutation levels may indicate increased survival prior to GC exit. Reduced mutations would point to accumulation while awaiting DZ re-entry.

Our studies measuring Caspase 3/7 and BrdU uptake did not document any changes in apoptosis or proliferation between WT and Itch KO GC B cell in either LZ or DZ. Due to the non-synchronized nature of the GC responses, it is still possible that Itch may regulate a survival checkpoint in LZ return or LZ selection, or a proliferation checkpoint in the LZ. Experimental approaches that can follow individual GC B cells through a GC cycle would be required to answer this question. One such study used the Dec-205 antigen delivery system to induce synchronized T cell help. After delivery of T cell help to GC B cells, there was an early increase in LZ/DZ ratio, followed by a sharp decrease (due to DZ expansion), then a return to starting LZ/DZ ratios after 4 days (13). In our previous study, we injected equal numbers of proliferation dye-labeled B1-8 GC cells into previously NP- immunized mice with active GCs, which resulted in synchronized GC proliferation(36). Future studies should take similar approaches to determine whether Itch limits apoptosis or proliferation in LZs.

It is important to note that our studies rely heavily on the model antigen system, NP-ova. Even in non-transgenic B6 mice, the B cell response to NP is dominated by a single IGHV gene (IGHV1-72). In studies using the transgenic B1-8 cells, we are essentially modeling intracolonal competition within GCs, losing the effect of a diverse clonal repertoire to a complex antigen (53). Future studies should move beyond hapten-carrier responses to determine how Itch regulation of LZ proliferation impacts humoral immune responses to complex antigens.

Our results also highlight the importance of post-translational modifications in controlling the dynamics of B cells navigating the germinal center. Only a few studies have begun to explore the role of directed proteolysis in GC biology. Due to the necessity for GC cells to make life and death decisions rapidly in response to extracellular cues, it seems plausible that ubiquitin-dependent proteolysis would help dictate these decisions. Surviving cells would then be governed by contributions from slower-acting transcriptional regulation, for example sustained proliferation in DZs triggered by Myc transcriptional activity. GC B cells are pre-programmed to die, such that survival cues rescue them from intrinsic apoptosis, mediated by mitochondrial changes(3, 54). Our proteomics analysis pointed to increased mitochondrial protein levels. Future studies will determine if Itch tips the balance on B cell life-death decisions through increased mitochondrial capacity, which may raise the threshold for intrinsic apoptosis, even with weak or delayed positive selection signals.

Taken together, our studies demonstrate that Itch regulates the way that GC B cells respond to antigenic stimulation and T cell help. This role limits hallmarks of positive selection, accumulation of LZ cells, and ultimately reduces the numbers of antigen-specific GC B cells. Through this role, Itch may help enforce Tfh cell dependent focused selection of the GC-diversified B cell repertoire.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Points

1. The ubiquitin ligase Itch limits the way B cells respond to the GC environment
2. Itch restricts accumulation of light zone GC B cells
3. Itch deficient GC B cells require less Tfh cell help

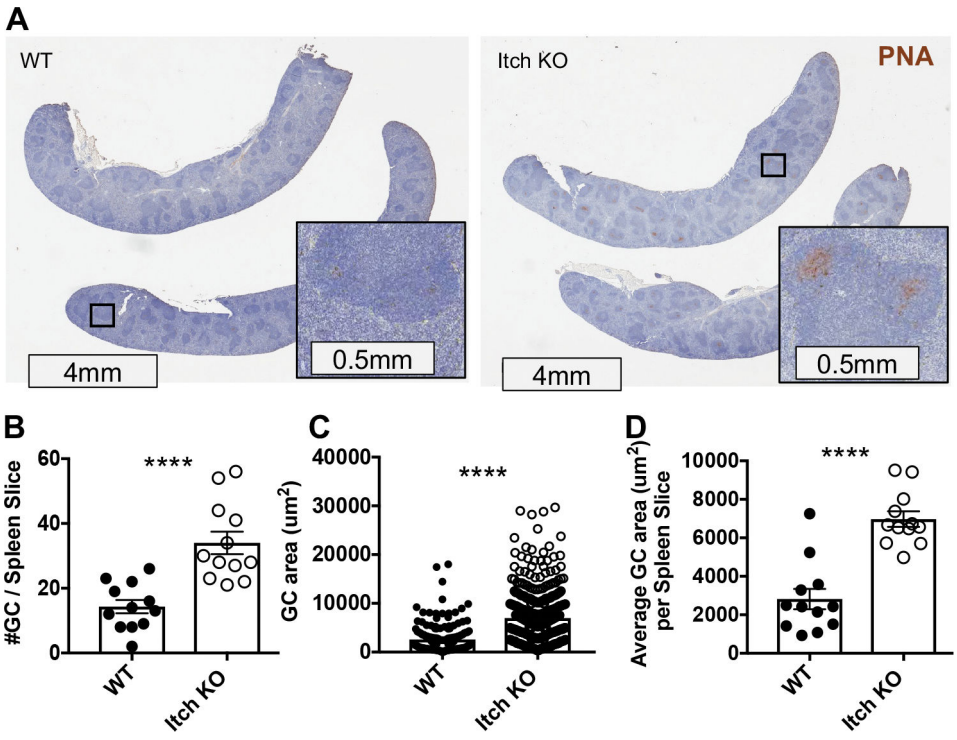

Figure 1: Unimmunized Itch deficient mice have larger and more numerous GCs

Spleens were harvested from 9–10 week old WT and Itch KO mice. Formalin-fixed, paraffin-embedded spleens were stained for PNA by immunohistochemistry. A) representative spleen sections are shown. 3 sections from each mouse were scanned, and GCs were circled using Aperio Image Scope software. Quantifications are shown of B) the number of GCs per spleen slice C) the area of individual GCs within all slices, and the average area of GCs per slice (n=4 mice, 3 slices per mouse, t-test, error bars represent SEM, * and ** denote $p<0.05$ and 0.01, respectively).

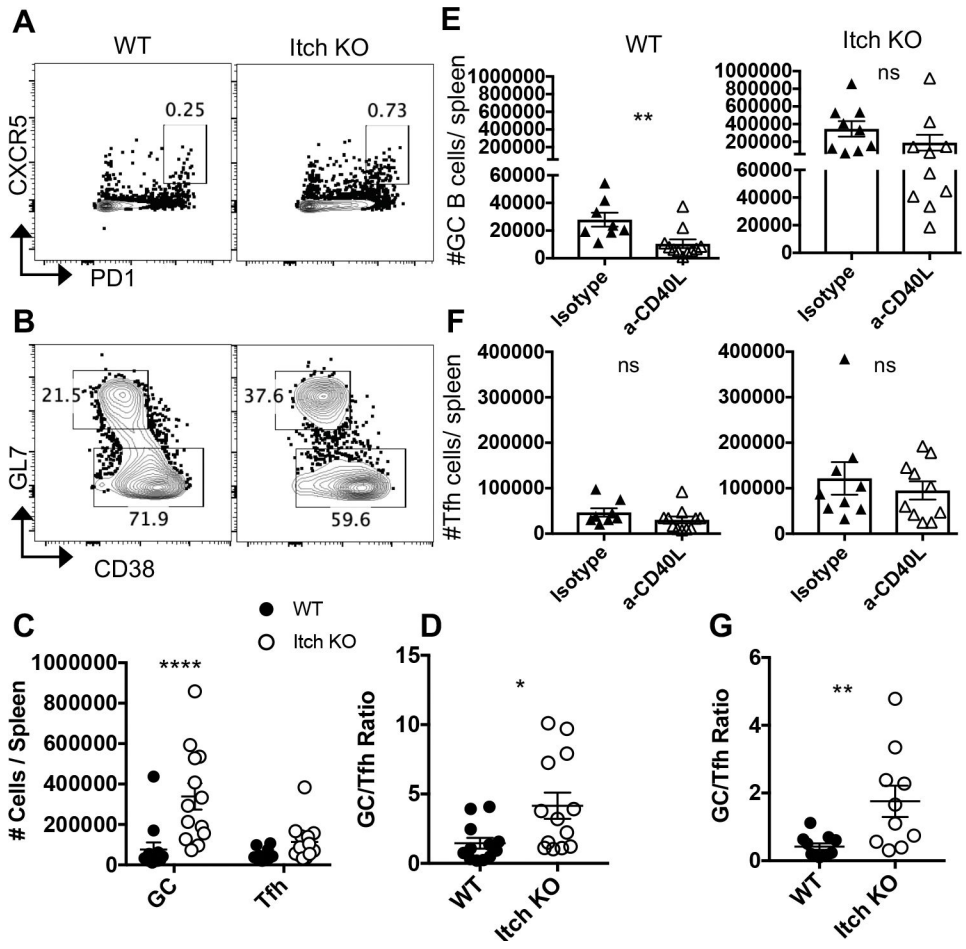

Figure 2: Itch deficient GC B cells are sustained by fewer Tfh

Spleens were harvested from 8–10 week old WT and Itch KO mice. A) Representative flow cytometry plots of Tfh cells. Cells were gated on live>singlet>CD3+CD4+. B) Representative flow cytometry plots of GC B cells. Cells were gated on live>singlet>IgD-IgM->CD19+. C) quantification of Tfh and GC absolute numbers from WT and Itch KO spleens (n=12–13, Tfh and GCs were paired within each mouse, 2-way ANOVA with Sidak post-tests). D) Within each mouse, the ratio of GC/Tfh cells is shown (n=12–13, t-test). E-F) WT and Itch KO mice treated with 200ug anti-CD40L or isotype on Day 0 and Day 2, and spleens were harvested on Day 3. E) GC B cells were quantified from spleens of WT and Itch KO mice. F) Tfh cells were enumerated from spleens of WT and Itch KO mice. G) Within each mouse, the ratio of GC/Tfh cells is shown. For E-G, n=8–10, t-test, error bars represent SEM. (*, **, ***, and **** denote $p<0.05$, 0.01, 0.001, and 0.0001, respectively).

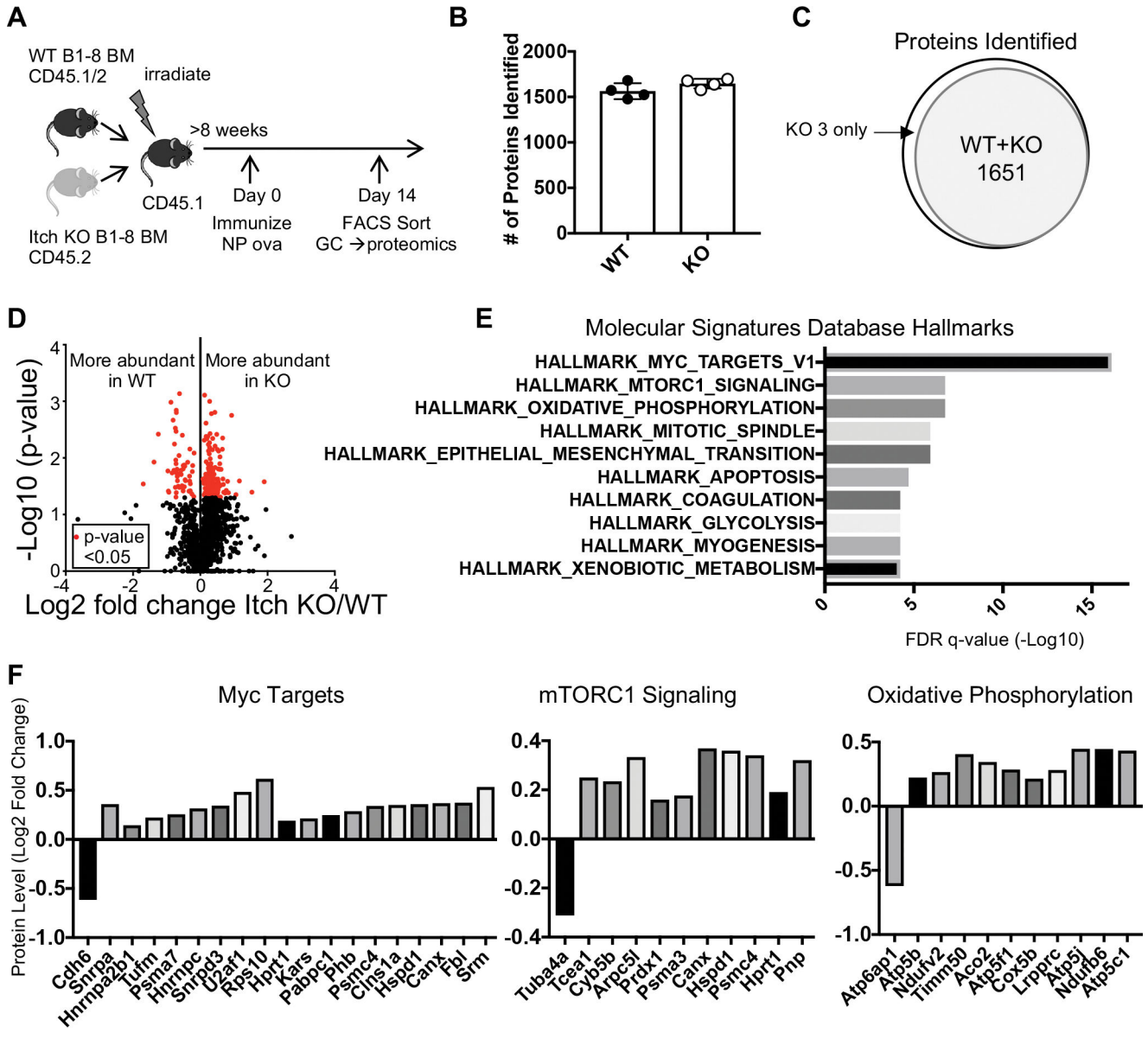

Figure 3: Itch KO GC cells show evidence of increased Myc, mTORC1, and oxidative phosphorylation

A) Mixed bone marrow chimeras were generated. CD45.1 mice were lethally irradiated and then transplanted with a 1:1 mixture of WT B1-8 (CD45.1/2) and Itch KO B1-8 (CD45.2) bone marrow by i.v. injection. After 8 weeks, mice were immunized with NP-ova in alum. On day 14 post immunization, WT and Itch KO GC cells (IgM-IgD-CD19+GL7+) were sorted by flow cytometry and then subject to proteomics analysis. B) Numbers of proteins identified in each sample. C) Overlap of protein identities between WT and Itch KO groups (error bars represent SEM). D) Quantification of protein abundance expressed as the Log2 fold change of Itch KO / WT. Paired t-test was performed (where proteins from WT and Itch KO cells were paired from each chimera). Red dots indicate $p<0.05$, n= 4. E) Differentially regulated proteins were subject to bioinformatic analysis using the Molecular Signatures

Database Hallmarks. Top 10 Hallmarks are shown. F) Protein abundances for each of the proteins within the top 3 Hallmarks are shown.

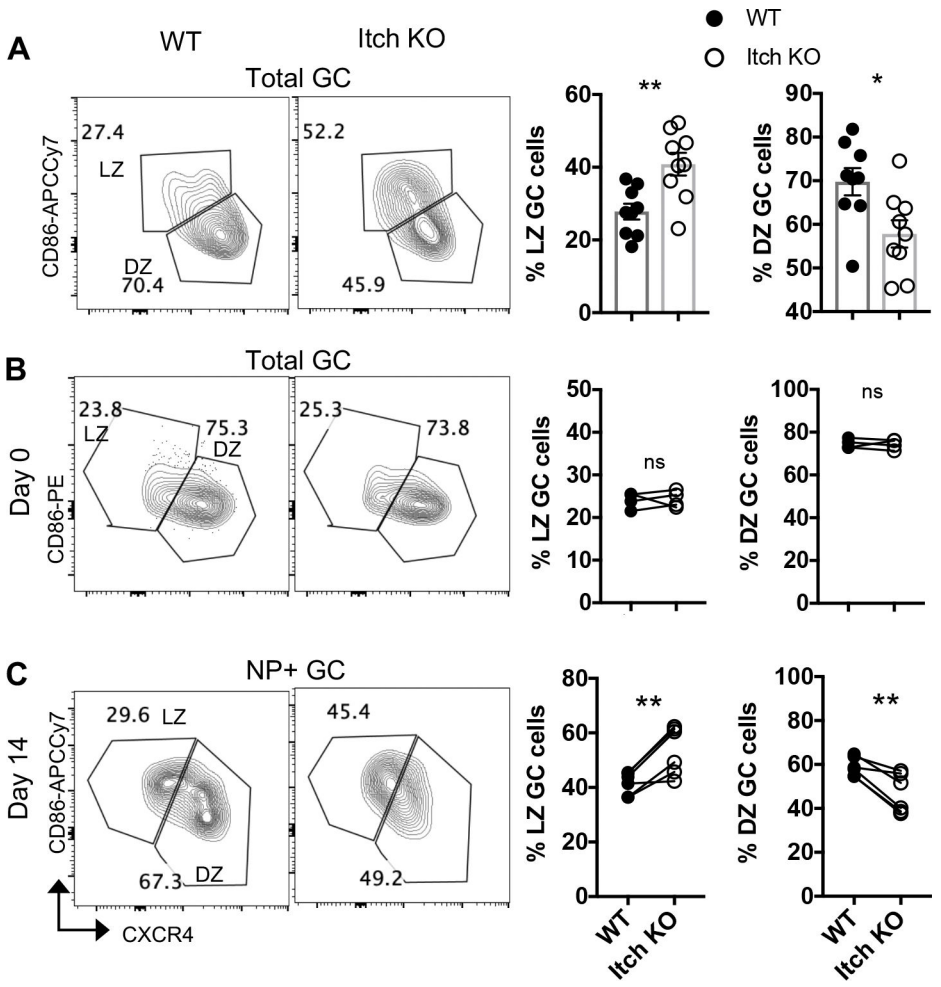

Figure 4: Itch deficiency increases proportion of LZ GC cells in presence of antigen.

A) WT and Itch KO splenic B cells were stained for LZ and DZ GC surface markers and analyzed by flow cytometry. Quantification of LZ and DZ percentages. Cells are gated on singlets>live>IgM^{-}IgD^{-}>CD19^{+}>CD38^{lo}GL7^{+}(n=9, t-test, error bars represent SEM). B-C) WT B1-8 and Itch KO B1-8 cells from spleens of mixed chimeras (described in Figure 3A) were analyzed for LZ and DZ GCs before (B) or after (C) immunization with NP-ova/alum. Cells are gated on singlets>live>IgM^{-}IgD^{-}>CD19^{+}>CD38^{lo}GL7^{+} and either CD45.1/2 (WT) or CD45.2 (Itch KO). In (C), GC cells are gated on NP+. (For Day 0: n=4, for Day 14, n=6. Paired t-test, * and ** denote $p<0.05$ and 0.01, respectively).

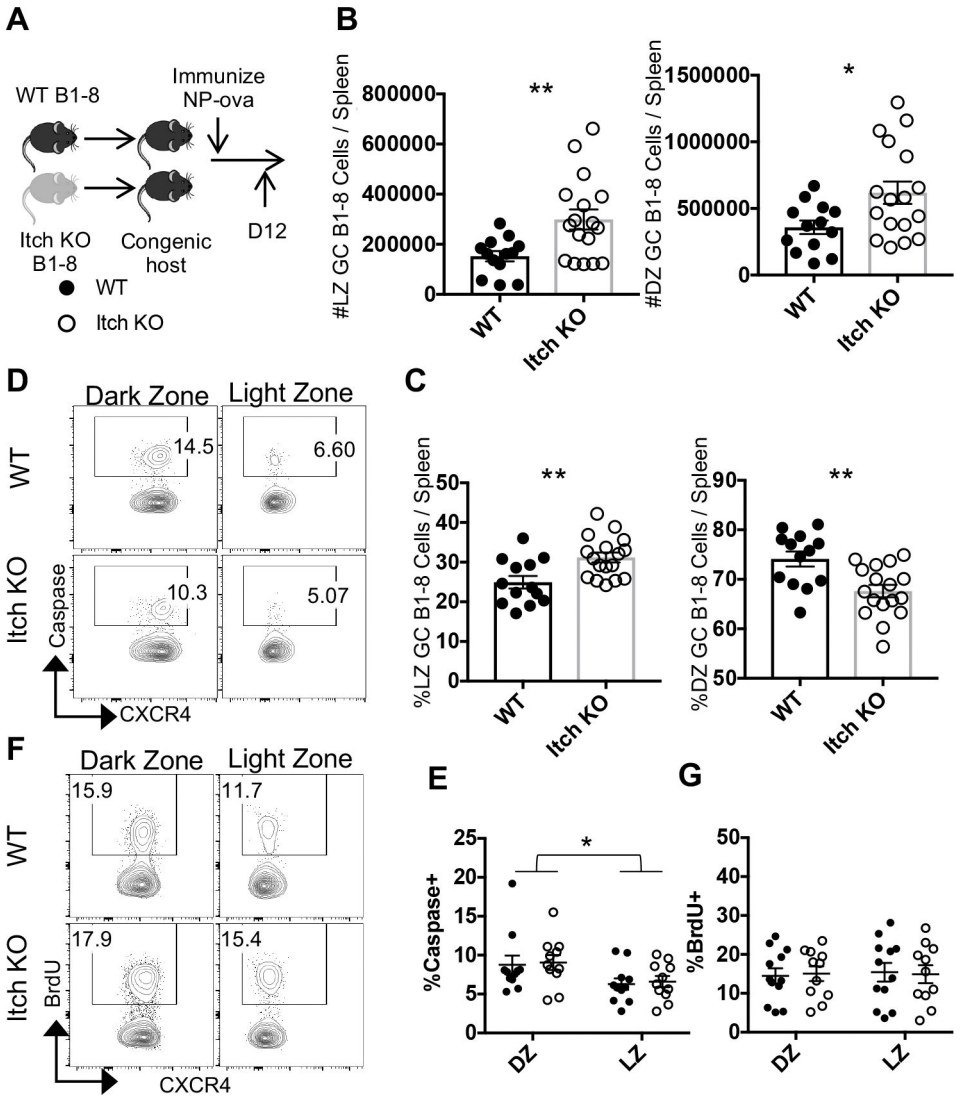

Figure 5: Itch deficient cells show higher LZ proportion in B1-8 adoptive transfer model

A) Experimental design for adoptive transfer. 20,000 WT or Itch KO lambda+ B1-8 cells (CD45.2) were transferred to WT congenic (CD45.1) mice then immunized with NP-ova/alum. B) Absolute numbers of splenic CD45.2 B1-8 NP-binding GC cells were quantified on day 12 post-immunization. Total GC cells, LZ, and DZ cells are shown. C) LZ and DZ percentages in WT and Itch KO B on GC cells (gated on live>singlet>IgM-IgD->CD19+>CD38loGL7+>CD45.2^{+}CD45.1^{-}) (n=13–17, data are pooled from 5 independent experiments, t-test, * and ** denote $p<0.05$ and 0.01, respectively, error bars represent SEM). D-E) Activated caspase 3/7 was measured in CD45.2+ LZ and DZ B1-8 GC cells by flow cytometry (n=11, data are pooled from 4 independent experiments, 2-way ANOVA, * $p<0.05$). Representative flow cytometry plots (D) and quantification (E) are shown. F-G) Recipient mice were injected i.p. with 1mg BrdU on Day 12 post adoptive transfer and immunization. One hour later, mice were sacrificed and spleen cells were stained for BrdU incorporation. Representative flow cytometry plots (F) and quantification (G) are shown (n=11–12, data are pooled from 4 independent experiments).

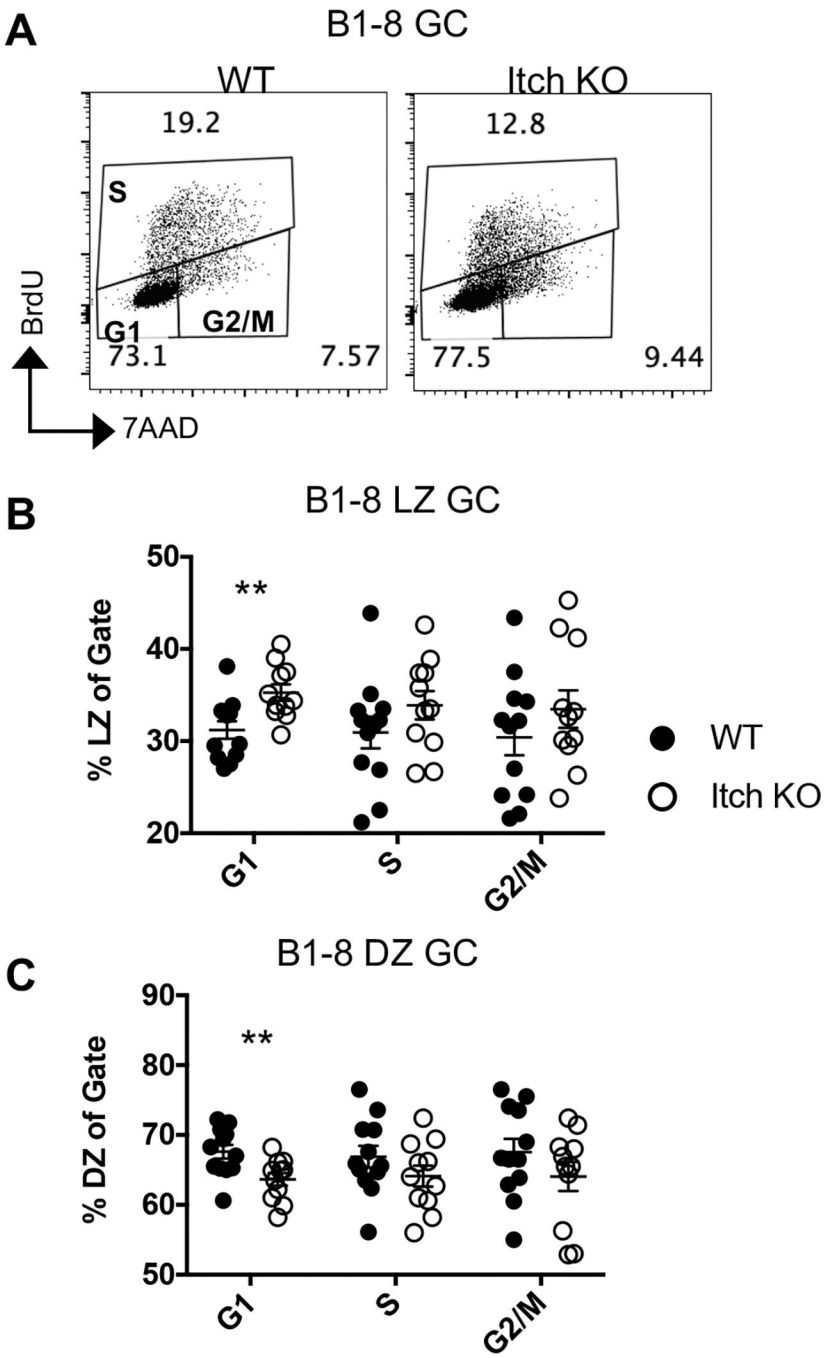

Figure 6: Itch limits cell accumulation of G1 cells in the LZ

20,000 WT or Itch KO lambda+ B1-8 cells (CD45.2) were transferred to WT congenic (CD45.1) mice then immunized with NP-ova/alum. Recipient mice were injected i.p. with 1mg BrdU on Day 12 post adoptive transfer and immunization. One hour later, mice were sacrificed and spleen cells were stained for BrdU incorporation and DNA content. A) Cells were gated on B1-8 GCs, and cell cycle gates were drawn based on DNA content (7AAD staining) and BrdU+ incorporation. B-C) Within each gate (G1, S, and G2/M) the percents

of B) LZ cells and C) DZ cells were enumerated. (n=11–12, 4 independent experiments, multiple t-tests, error bars represent SEM).