Follicular helper T cells are required for systemic autoimmunity

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Production of high-affinity pathogenic autoantibodies appears to be central to the pathogenesis of lupus. Because normal high-affinity antibodies arise from germinal centers (GCs), aberrant selection of GC B cells, caused by either failure of negative selection or enhanced positive selection by follicular helper T (T FH ) cells, is a plausible explanation for these autoantibodies. Mice homozygous for the san allele of Roquin, which encodes a RING-type ubiquitin ligase, develop GCs in the absence of foreign antigen, excessive T FH cell numbers, and features of lupus. We postulated a positive selection defect in GCs to account for autoantibodies. We first demonstrate that autoimmunity in Roquin san/san (sanroque) mice is GC dependent: deletion of one allele of Bcl6 specifically reduces the number of GC cells, ameliorating pathology. We show that Roquin san acts autonomously to cause accumulation of T FH cells. Introduction of a null allele of the signaling lymphocyte activation molecule family adaptor Sap into the sanroque background resulted in a substantial and selective reduction in sanroque T FH cells, and abrogated formation of GCs, autoantibody formation, and renal pathology. In contrast, adoptive transfer of sanroque T FH cells led to spontaneous GC formation. These findings identify T FH dysfunction within GCs and aberrant positive selection as a pathway to systemic autoimmunity.

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pathway and form germinal centers (GCs) (4). Within this microenvironment, B cells undergo somatic hypermutation (SHM) and isotype switching, resulting in the generation of memory B cells and long-lived plasma cells that secrete high-affinity antigen-specific IgG antibodies (5, 6). Selection of mutated high-affinity GC B cells depends on restimulation with antigen arrayed on follicular dendritic cells and provision of help by follicular T helper (T<sub>FH</sub>) cells.

Because SHM has the potential to generate self-reactive antibodies (7), it has been long thought that aberrant selection within GCs represents a candidate pathway to the production of lupus-associated autoantibodies. Indeed, autoantibodies detected in SLE patients and mouse lupus models are generally high affinity and somatically mutated (7, 8). Exclusion of self-reactive B cells from GCs has been shown to be defective in SLE patients. Also, GCs have been shown to form spontaneously in several different mouse models of lupus (9), and these are rich in apoptotic cells displaying the antigenic targets of lupus autoimmunity (10, 11). Although SHM can occur outside GCs, this process is far less efficient (12, 13). Despite all of this circumstantial evidence, there is to date no definite proof that GCs and/or T<sub>FH</sub> cells are directly required for the production of lupus autoantibodies or end-organ damage. In contrast, extrafollicular affinity maturation of autoantibodies to dsDNA in MRL<sup>Ab</sup> mice (14, 15) and T-independent B cell activation factors of the TNF family-driven pathways to lupus characteristics of polynia, and glomerulonephritis with IgG-containing immune lymphanedopathy, splenomegaly, autoimmune thrombocytopenia, and exhibits a lupus-like phenotype characterized by high-affinity anti-dsDNA antibodies, hypergammaglobulinemia, lymphadenopathy, splenomegaly, autoimmune thrombocytopenia, and glomerulonephritis with IgG-containing immune complex deposits. Both the autoimmunity and cellular characteristics of sanroque segregate with homozygosity for the san allele (M<sub>19</sub>R substitution) of Roquin (Roquin<sup>Cts</sup>/san) (22). Roquin has been demonstrated to be a regulator of the stability T cell messenger RNAs. Development of autoimmunity in Roquin<sup>Cts</sup>/san mice also correlates with spontaneous GC formation, which is largely driven by B cell–extrinsic factors (22). Roquin<sup>Cts</sup>/san mice have a marked accumulation of T cells within the B cell follicles, and the T<sub>FH</sub> subset is overrepresented within the CD4<sup>+</sup> cell compartment.

In this paper, we report that dysregulation of the GC response through excessive formation of T<sub>FH</sub> cells is responsible for autoimmunity in Roquin<sup>Cts</sup>/san mice. Loss of one allele of B6.6, the master transcriptional regulator of GCs (25, 26), significantly reduces spontaneous GC formation in Roquin<sup>Cts</sup>/san mice and the lupus phenotype. Furthermore, deletion of Sap (Sh2d1a) from Roquin<sup>Cts</sup>/san mice causes a dramatic reduction of T<sub>FH</sub> cells and IL-21. Sap is a small adaptor protein necessary for signaling through the signaling lymphocyte activation molecule family cell-surface receptors that regulates signals downstream of the TCR. Roquin<sup>Cts</sup>/san Sap<sup>+/−</sup> CD4<sup>+</sup> cells also express lower levels of ICOS than Roquin<sup>Cts</sup>/san Sap<sup>+/+</sup> cells. This results in abrogation of ANAs (including anti-dsDNA) and end-stage renal disease. These findings establish a causal pathway from the san allele of Roquin to excess T<sub>FH</sub> formation, aberrant GC formation, and positive selection of pathogenic high-affinity autoantibodies to illuminate a novel pathway of lupus pathogenesis.

RESULTS

**Heterozygous Bcl6 deficiency reduces spontaneous GCs and attenuates autoimmunity in Roquin<sup>Cts</sup>/san mice**

BCL6 has been shown to be the master transcriptional regulator of GC B cells (27). As BCL6 deficiency results in early mortality because of widespread inflammation, we investigated Bcl6<sup>+/−</sup> mice for possible defects in the GC response. 8 d after sheep red blood cell (SRBC) immunization, the percentage of GC cells was more than fivefold lower in mice heterozygous for B6.6 deficiency (2.3 ± 0.59% vs. 0.43 ± 0.46%; P = 0.0011; Fig. 1 a).

To determine whether B6.6 heterozygosity could curtail the spontaneous GC response in Roquin<sup>Cts</sup>/san mice, we compared the percentage of GC cells in unimmunized Roquin<sup>Cts</sup>/san Bcl6<sup>+/−</sup> and Roquin<sup>Cts</sup>/san Bcl6<sup>+/−</sup> mice. As seen in wild-type mice, loss of one allele of B6.6 caused a twofold reduction (4.4 ± 1.9% vs. 2.1 ± 0.5%; P = 0.0026) in the percentage of spontaneous GCs in Roquin<sup>Cts</sup>/san mice (Fig. 1 b). Loss of one allele of Bcl-6 also reduced GC B cells after SRBC immunization in a cell-autonomous manner (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20081886/DC1). We then tested whether reduction in the spontaneous GC response in Roquin<sup>Cts</sup>/san mice was accompanied by reduced pathology. Serum dsDNA antibodies were present in 50% of Roquin<sup>Cts</sup>/san Bcl6<sup>+/−</sup> mice tested compared with 100% of Roquin<sup>Cts</sup>/san Bcl6<sup>+/−</sup> mice (Fig. 1 c). Kidney pathology was also significantly reduced in Roquin<sup>Cts</sup>/san Bcl6<sup>+/−</sup> mice (Fig. 1, d and e).

**The lupus-like pathology of Roquin<sup>Cts</sup>/san mice requires T cell activation**

We have previously demonstrated that Roquin acts predominantly B cell extrinsically to induce spontaneous GC formation,
and T cell intrinsically to prevent accumulation of activated/memory T cells and repress ICOS expression (22, 28). These findings suggest that the spontaneous GC reaction is driven by Roquin-mediated dysregulation of T cells. In a first attempt to confirm that Roquin′/+ lupus T cells are T cell driven, we generated Roquin′/+ mice deficient in the major T cell co-stimulator CD28 (CD28–/– mice). Failure to prime T cells in Roquin′/+ mice abrogated the production of antinuclear IgG, including high-affinity anti-dsDNA antibodies (Fig. 2 a). Both interstitial nephritis and glomerular pathology, characterized by increased mesangial cellularity and dense deposits observed in 6-mo-old Roquin′/+ mice, were substantially reduced in age-matched Roquin′+/+ CD28–/– mice, with only very mild interstitial nephritis (Fig. 2, b and c). Less than half of the Roquin′/+ mice examined had low-grade mesangial immune complex deposits (Fig. 2 c). Of note, Roquin′/+ CD28–/– naive CD4+ T cells had a twofold decrease in ICOS expression (29).

**Expansion of the Tfh subset in Roquin′+/− mice is T cell intrinsic**

We have previously reported the similarity of the gene expression profiles of Roquin′+/− CD4+ T cells and Tfh cells. Furthermore, Roquin′+/− T cells accumulate in the splenic GCs (23). To formally assess whether Tfh cells, defined as CD4+CXCR5+PD−1high (30), are expanded in Roquin′+/− mice, we analyzed the percentage and total number of these cells in unimmunized mice and found more than a threefold increase of this subset in Roquin′+/− mice compared with littermate controls (Fig. 2 d). To determine whether this aberrant Tfh cell accumulation is cell intrinsic, we generated mixed chimeras. Sublethally irradiated Roquin′+/Ly5b mice were reconstituted with a 1:1 mix of Roquin′−/Ly5b and Roquin′+/Ly5b bone marrow cells. As a control, Roquin′+/Ly5b mice were reconstituted with a 1:1 mix of Roquin′−/Ly5b and Roquin′−/Ly5b bone marrow cells. 8 wk after reconstitution, mice were immunized with SRBCs, and the percentage of Tfh cells derived from each type of donor marrow was determined by flow cytometry. In chimeras reconstituted with Roquin′+/Ly5b/Roquin′−/Ly5b or Roquin′+/Ly5b/Roquin′+/Ly5b marrow, three times more Ly5b (Roquin′+/−) Tfh cells than Ly5b cells were observed (P = 0.001), whereas in controls, an equivalent proportion of Tfh cells arose from Ly5b and Ly5b cells (P = 0.56; Fig. 2 e). This indicates that Roquin acts in T cells to repress the formation and/or survival of Tfh cells. Consistent with the observed increase in Tfh cell numbers, IL-21 production was more than twofold higher in splenocyte cultures from Roquin′+/− mice (Fig. 2 f).

Although Tfh cells are expanded in Roquin′+/− mice, it was important to exclude defects in other T cell subsets implicated in autoimmunity. CD4+CD25+FoxP3+ T reg cells have been shown to play a role in the regulation of lupus-associated autoantibodies (31). T reg cells are not reduced in number or function in Roquin′+/− mice (22). Quantification of FoxP3+CD25+CD4+ cells in the spleen confirmed previous observations: Roquin′+/− mice have approximately twofold more T reg cells than wild-type mice (18.9 ± 3.69% vs. 9.76 ± 1.03%, respectively; Fig. S2 a, available at http://www.jem.org/cgi/content/full/jem.20081886/DC1). This argues against a role for reduced T reg cell numbers in driving the T cell–mediated disease of Roquin′+/− mice. Another T helper subset, Th17 cells, have emerged as potent mediators of autoimmunity (31), and recent work suggests that they may be critical to maintain the spontaneous GCs of B6D2 mice (23). To test whether Th17 cell activity is dysregulated in Roquin′+/− mice, we analyzed IL-17 levels in splenocyte cell cultures after activation with PMA and ionomycin. IL-17 was detected at comparable levels in cultures from Roquin′+/− and Roquin′+/+ mice (Fig. S2 b). In contrast, IL-21, a cytokine secreted by both Tfh and Th17 cells (33, 34), was found at significantly higher levels in Roquin′+/− splenocyte cultures (Fig. 2 f). Collectively these data indicate that the Tfh subset in Roquin′+/− mice is expanded in a cell-autonomous manner, whereas T reg and Th17 cells do not appear to be dysregulated in a way that has been previously described to result in autoimmunity.

**SAP−deficient mice form reduced numbers of Tfh cells**

Having established that Roquin acts T cell intrinsically to dysregulate Tfh cell numbers leading to the formation of abundant GCs, we hypothesized that disrupting the GC reaction through selective reduction of Tfh cell numbers or function would attenuate systemic autoimmunity. SAP is an adaptor protein necessary for signaling through the signaling lymphocyte activation molecule family receptors that regulates signals downstream of the TCR. Mice lacking SAP cannot form GCs or generate immunological memory after immunization, a result of impaired CD4+ T cell help to GC B cells after antigen exposure (35–38). To date, it is not known whether SAP signaling is also required to maintain optimal Tfh cell numbers. We enumerated CD4+CXCR5+PD−1high Tfh cells in SAP−deficient unimmunized mice and wild-type littermates, and observed a twofold decrease in the number of basal Tfh cells (Fig. 3 a, left). This was accompanied by a fourfold reduction in the number of background GC B cells (Fig. 3 b, right).

**Roquin′+/− Sap−/− mice do not form excessive Tfh cells or spontaneous GCs**

Once we had established that SAP deficiency not only impairs CD4+ T cell help for GC B cells but also decreases Tfh cell numbers, we generated Roquin′+/− Sap−/− mice to determine whether numerically and functionally defective Tfh cells in Roquin′+/− mice could abrogate the spontaneous GC reactions. As observed in SAP−deficient mice, loss of SAP in Roquin′+/− mice also had an effect on Tfh cell numbers: there was a fourfold reduction (0.94 ± 0.41% vs. 3.59 ± 1.99%) in the percentage of Tfh cells in Roquin′+/− Sap−/− mice relative to Roquin′+/− mice (P = 0.0056; Fig. 3 a, middle). SAP deficiency also resulted in a 10-fold reduction in the percentage of GC B cells in Roquin′+/− Sap−/− mice (4.27 ± 1.93% vs. 0.43 ± 0.23%; P = 0.0007; Fig. 3 b, middle). Immunohistochemistry paralleled the flow cytometry data, showing reduced size and number of GCs, and a reduction of T cell numbers within PNA-positive follicles (Fig. 3 c). These findings indicate that loss of SAP corrects the aberrant formation of Tfh cells and the
Figure 1. Heterozygosity for Bcl6 reduces the magnitude of the GC response in Roquin<sup>+/+</sup> and Roquin<sup>san/san</sup> mice and ameliorates the lupus-like phenotype of Roquin<sup>san/san</sup> mice. (a) Flow cytometric contour plots (left) and graphical analysis (right) of B220<sup>+</sup>GL-7<sup>+</sup>CD95<sup>+</sup> GC B cells in 10-wk-old wild-type (Bcl6<sup>+/+</sup>) and Bcl6<sup>+/−</sup> mice 8 d after SRBC immunization (P = 0.0011). Data are representative of four independent experiments (n = 4 per group). (b) Flow cytometric contour plots (left) and dot plots (right) showing B220<sup>+</sup>GL-7<sup>+</sup>CD95<sup>+</sup> GC B cells from 10-wk-old naive Roquin<sup>san/san</sup> Bcl6<sup>+/+</sup> and Roquin<sup>san/san</sup> Bcl6<sup>+/−</sup> mice. Data are representative of five independent experiments (n ≥ 4 per group). (c) Representative determination of serum IgG anti-dsDNA from 6-mo-old female Roquin<sup>+/+</sup> Bcl6<sup>+/+</sup>, Roquin<sup>san/san</sup> Bcl6<sup>+/+</sup>, and Roquin<sup>san/san</sup> Bcl6<sup>+/−</sup> mice, determined by immunofluorescence staining of C. luciliae substrate. Data shown reflect the occurrence (n ≥ 6 mice per group); three out of six Roquin<sup>san/san</sup> Bcl6<sup>+/−</sup> mice had low intensity staining (illustrated in the fourth panel from left).
inappropriate GC reaction phenotype of Roquin\(\text{an/an}\) to levels comparable to Roquin\(+/+\) mice.

To test whether SAP alters other T effector subsets in sanroque mice, we quantified Th1 and Th2 cells by flow cytometric staining for Tbet and Gata3, respectively. There were no statistically significant differences in the percentage of CD44\(\text{high}\)/Gata3\(\text{CD4}^+\) cells (0.91 ± 0.14% vs. 1.15 ± 0.41%; \(P = 0.18\)) or CD44\(\text{high}\)/Tbet\(\text{CD4}^+\) cells (6.76 ± 10.6% vs. 8.66 ± 5.74%; \(P = 0.59\)) between Roquin\(\text{an/an}\)/Sap\(+/-\) and Roquin\(\text{an/an}\)/Sap\(^-/-\) mice in peripheral blood (Fig. 4, a and b). Lymph node CD44\(\text{high}\)/Gata3\(\text{CD4}^+\) cells (2.11 ± 1.68% vs. 1.59 ± 0.81%; \(P = 0.54\)) and CD44\(\text{high}\)/Tbet\(\text{CD4}^+\) cells (2.89 ± 1.9% vs. 2.02 ± 0.64%; \(P = 0.25\)) between Roquin\(\text{an/an}\)/Sap\(+/-\) and Roquin\(\text{an/an}\)/Sap\(^-/-\) mice were slightly reduced, but these differences were not statistically significant (Fig. 4 c). In contrast, SAP deficiency caused an approximately fourfold reduction in Roquin\(\text{an/an}\) lymph node T\(\text{FH}\) cells (3.93 ± 0.47% vs. 1.11 ± 0.38%; \(P = 0.001\); Fig. 4 c). Collectively, these data indicate that T\(\text{FH}\) cells are the T helper subset whose generation is most severely impaired by SAP deficiency in Roquin\(\text{an/an}\) mice.

**Transfer of Roquin\(\text{an/an}\) T\(\text{FH}\) cells induces spontaneous GC reactions in wild-type mice**

To test whether Roquin\(\text{an/an}\) T\(\text{FH}\) cells are sufficient to induce GC reactions in immunized wild-type mice, CD45.2\(\text{PD-1}^\text{high}\)/CXCR5\(\text{PD-1}^\text{high}\)/CD4\(^+\) T\(\text{FH}\) cells or CD45.2\(\text{CXCR5}^\text{PD-1}^\text{high}\)/CD4\(^+\) non-T\(\text{FH}\) T effector cells were adoptively transferred into CD45.1\(\text{C57BL/6}\) mice. 3 wk after transfer, 8% of donor T\(\text{FH}\) cells retained their CXCR5\text{high} phenotype. In mice receiving non-T\(\text{FH}\) san/san CD4-44\text{high} effectors, ~3% of the transferred cells had also acquired a comparable T\(\text{FH}\) phenotype (Fig. 4 d). Adoptive transfer of Roquin\(\text{an/an}\) T\(\text{FH}\) cells resulted in a threefold increase in the number of GC B cells compared with control mice injected with PBS alone (\(P = 0.003\); Fig. 4, e and f). A small increase in the percentage of GC cells was also observed in the mice that received non-T\(\text{FH}\) effector cells, although this was not statistically significant (Fig. 4, e and f). These data suggest that Roquin\(\text{an/an}\) T\(\text{FH}\) cells can drive a GC reaction in the absence of exogenous antigen.

**SAP deficiency abrogates the lupus-like phenotype of Roquin\(\text{an/an}\) mice**

Having established that SAP deficiency prevents T\(\text{FH}\) cell accumulation and selectively impairs GC responses, we sought to determine whether SAP deficiency affects the serum autoantibody and renal pathology of Roquin\(\text{an/an}\) mice. Roquin\(\text{an/an}\)/Sap\(+/-\) mice had not developed ANAs by 8 wk of age (Fig. 5 a). Assessment of high-affinity anti-dsDNA IgG antibodies at 6 mo of age (by immunofluorescence using *Crithidia luciliae* substrate) revealed positive dsDNA antibodies in all Roquin\(\text{an/an}\) mice evaluated, whereas only one out of six Roquin\(\text{an/an}\)/Sap\(+/-\) mice had detectable antibodies. Renal histology revealed that Roquin\(\text{an/an}\)/Sap\(+/-\) mice had only minor interstitial nephritis and thickening of the mesangial matrix, with no detection of immune complex deposition observed by electron microscopy (Fig. 5, b and c). These data suggest that abrogation of signaling through SAP greatly reduced the autoimmune manifestations of Roquin\(\text{an/an}\) mice. In contrast, SAP deficiency conferred no significant reduction of splenomegaly, lymphadenopathy, and hypergammaglobulinemia present in Roquin\(\text{an/an}\) mice, indicating that these defects are not mediated by excessive T\(\text{FH}\) cell formation and GC function (Fig. S3, a–c, available at http://www.jem.org/cgi/content/full/jem.20081886/DC1).

**Reduction of the GC response in Roquin\(\text{an/an}\)/Sap\(+/-\) mice is mainly caused by B cell–extrinsic factors**

Several studies have shown that SAP acts T cell intrinsically to regulate GC responses (35, 37, 39), but some controversy still exists in the light of one study showing B cell–intrinsic SAP-mediated effects (40). To test whether the correction of the GC response observed in Roquin\(\text{an/an}\)/Sap\(+/-\) mice was caused by B cell–extrinsic factors, we transferred SW HEL B cells expressing intact SAP into Roquin\(\text{+/-}\)/Roquin\(\text{+/-}\)/Sap\(+/-\) and Roquin\(\text{an/an}\)/Sap\(+/-\) mice, in conjunction with hen egg lysozyme (HEL)\text{2x}–conjugated SRBCs.

Adaptively transferred SW HEL B cells formed HEL-specific GCs in all four cohorts. Significantly fewer GCs were observed in Sap\(+/-\) than in wild-type mice (23,958 ± 15,920 vs. 1,186 ± 1,019; \(P = 0.005\); Fig. 5 d), confirming a B cell–extrinsic contribution of SAP to the GC response (34, 36, 38). In addition, SAP deficiency corrected the extreme GC formation observed in Roquin\(\text{an/an}\) mice (124,528 ± 25,980 vs. 30,528 ± 18,100; \(P = 0.0004\)) to a response of similar magnitude to Roquin\(\text{+/-}\)/Roquin\(\text{+/-}\)/Sap\(+/-\) mice (Fig. 5 a). This suggests that the SAP-mediated effect in Roquin\(\text{an/an}\) mice is also caused by B cell–extrinsic factors, and is most likely caused by SAP’s regulation of T\(\text{FH}\) cells.

To determine whether the loss of Sap in Roquin\(\text{an/an}\) mice selectively affected the GC response or also impaired the extrafollicular response, we identified donor–derived extrafollicular plasma cells containing intracellular anti-HEL Ig at day 5 after immunization (Fig. 5 e). At this time point, GC-derived
Figure 2. The autoimmune phenotype of Roquin<sup>san/san</sup> mice requires T cell activation through CD28, and T<sub>HF</sub> cells are expanded cell auton-omously. (a, left) Detection of IgG-ANA using Hep-2 slides in sera from 8-wk-old female Roquin<sup>san/san</sup> and Roquin<sup>san/san</sup> Cd28<sup>-/-</sup> mice (n = 5 per group). (right) Detection of anti-dsDNA IgG serum antibodies in 6-mo-old female Roquin<sup>san/san</sup> and Roquin<sup>san/san</sup> Cd28<sup>-/-</sup> mice determined by staining C. luciliae slides. Data are representative of three independent experiments (n ≥ 5 per group). (b) Score of nephritis severity in 6-mo-old female Roquin<sup>+/+</sup>, Roquin<sup>san/san</sup> and Roquin<sup>san/san</sup> Cd28<sup>-/-</sup> mice as determined by histological analysis defined by the criteria given in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20081886/DC1). Each symbol represents one mouse. Horizontal bars indicate medians. (c) Representative images of kidney sections stained with H&E (left) or viewed under an electron microscope (right). Histology from Roquin<sup>san/san</sup> Cd28<sup>-/-</sup> animals was much less severe, with normal H&E appearances and few electron-dense deposits (arrows) in the mesangium. Bars: (left) 100 μm; (right) 10 μm. (d) Representative flow cytometric contour plots of CD4<sup>+</sup> CXCR5<sup>+</sup> PD-1<sup>high</sup> T<sub>HF</sub> cells in 10-wk-old Roquin<sup>san/san</sup> mice and control littermates. Data are representative of five independent experiments (n = 4 per group), and the numbers in the plots represent percentages. (e) Dot plots representing percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>PDX<sup>high</sup> T<sub>HF</sub> cells from SRBC-immunized chimeric mice generated by reconstituting sublethally irradiated mice with a 1:1 mix of either Roquin<sup>+/+</sup>Ly5<sup>+</sup>/Roquin<sup>san/san</sup>Ly5<sup>-</sup> (left) or Roquin<sup>san/san</sup>Ly5<sup>-</sup>/Roquin<sup>san/san</sup>Ly5<sup>-</sup> (right). Data are representative of three independent experiments (n = 4 per group). Each symbol represents the Ly5<sup>-</sup> or
plasma cells cannot be detected (41). Although we observed a 4-fold decrease in SWHEL GC B cells, there was only a 1.3-fold decrease in the number of SWHEL extracellular plasma cells (53,198 ± 1,528 vs. 40,528 ± 7,548; P = 0.04) in Roquin<sup>san/san</sup> Sap<sup>−/−</sup> recipients compared with Roquin<sup>san/san</sup> Sap<sup>+/+</sup> controls (Fig. 5e). These data suggest that the reduction of the GCs in Roquin<sup>san/san</sup> mice caused by Sap deficiency is mainly a result of B cell–extrinsic factors, and that this results in selective inhibition of the GC pathway.

**Roquin<sup>san/san</sup> Sap<sup>−/−</sup> mice have reduced expression of ICOS and IL-21**

We have demonstrated that Roquin<sup>san/san</sup> Sap<sup>−/−</sup> mice have fewer T<sub>FH</sub> cells, a correction of the spontaneous GC response, and a reduction in autoimmune pathology. To gain insight into the possible functional T<sub>FH</sub> defect brought about by SAP deficiency in Roquin<sup>san/san</sup> mice, we quantified the changes in the expression of ICOS and IL-21, both of which are increased in Roquin<sup>san/san</sup> CD4<sup>+</sup> cells (22) and are shown to be essential for T<sub>FH</sub> cell formation, homeostasis, and function (33, 34, 42, 43). Loss of SAP reduced the levels of ICOS on both naive (CD4<sup>4lo</sup>) and activated/memory (CD4<sup>4hi</sup>) CD4<sup>+</sup> T cells in Roquin<sup>san/san</sup> mice and to an even greater extent on Roquin<sup>+/+</sup> mice (Fig. 6a–c). Despite this reduction, Roquin<sup>san/san</sup> Sap<sup>−/−</sup> mice still expressed more than threefold higher levels of ICOS than Roquin<sup>+/+</sup> Sap<sup>+</sup> mice.

To determine whether loss of SAP had an effect on the production of IL-21, we compared IL-21 production from Roquin<sup>+/+</sup> and Roquin<sup>+/+</sup> splenocytes. SAP deficiency reduced IL-21 production in both Roquin<sup>+/+</sup> and Roquin<sup>+/+</sup> splenocytes (Fig. 6d). Remarkably, the levels of IL-21 found in Roquin<sup>+/+</sup> and Roquin<sup>+/+</sup> splenocytes were comparable to those of wild-type (Roquin<sup>+/+</sup> Sap<sup>+/+</sup>) mice as previously reported (37), SAP deficiency resulted in higher CD40L expression upon activation in both Roquin<sup>+/+</sup> and Roquin<sup>+/+</sup> mice (Fig. 6e and f).

**IL-21 deficiency does not prevent autoimmunity in Roquin<sup>san/san</sup> mice**

Given that Roquin<sup>san/san</sup> mice produce high levels of IL-21 and given the recently described role for IL-21 in T<sub>FH</sub> cell generation after immunization (33, 34), we investigated whether IL-21 also controls autoantibody production, T<sub>FH</sub> cell accumulation, and spontaneous GC formation in these mice. To this end, we crossed Roquin<sup>san/san</sup> mice to IL-21<sup>−/−</sup> mice on a C57BL/6 background and assessed ANA formation. There was no difference in the pattern or titer of ANAs produced by 10-wk-old Roquin<sup>san/san</sup> IL-21<sup>−/−</sup> and Roquin<sup>san/san</sup> IL-21<sup>−/−</sup> mice (Fig. 7a and b). Overall, IL-21 deficiency did not affect the hypergammaglobulinemia of Roquin<sup>san/san</sup> mice (Fig. 7c), and consistent with previous reports (44), IgG1 titers were reduced, whereas IgE titers were increased (Fig. 7c). Likewise, loss of IL-21 did not correct the lymphadenopathy or the splenomegaly of Roquin<sup>san/san</sup> mice (Fig. 7d).

We then investigated whether IL-21 influences T<sub>FH</sub> cell accumulation and spontaneous GC formation. There was no difference in either the number of T<sub>FH</sub> cells or GC cells between Roquin<sup>san/san</sup> IL-21<sup>+/+</sup> and Roquin<sup>san/san</sup> IL-21<sup>−/−</sup> littermates (Fig. 7e and f). These data suggest that IL-21 does not play a role in spontaneous GC formation, T<sub>FH</sub> development, or accumulation in Roquin<sup>san/san</sup> mice.

**DISCUSSION**

Elucidation of the defect that results in high-affinity antibodies to dsDNA is central to understanding lupus and, hence, the development of specific therapeutic interventions. In the Roquin<sup>san/san</sup> model, a fundamental GC defect results in the production of autoantibodies. This pathway appears to be critical for disease development, because an impediment to GC formation conferred by halving the dose of Btk is sufficient to attenuate the lupus phenotype. Our data are consistent with other evidence that aberrant T cell help can cause spontaneous GC formation and autoimmunity (9, 45). We show the expanded T<sub>FH</sub> cell subset, a consistent component of the Roquin<sup>san/san</sup> phenotype, is responsible for excessive GC formation. In Roquin<sup>san/san</sup> mice, GCs form in the absence of foreign antigen and yield ANAs. SAP deficiency reduces the size and activity of the T<sub>FH</sub> subset and abrogates autoantibody production and renal disease. These findings provide the in vivo cellular mechanism to link the defect in microRNA (miRNA)–mediated repression of T<sub>FH</sub> molecules conferred by the san allele of Roquin with the lupus phenotype of Roquin<sup>san/san</sup> mice (22, 28). Moreover, they elucidate a mechanism of lupus that could be generalized to other defects of T<sub>FH</sub> homeostasis. Importantly, they provide evidence that defects in positive selection of GC B cells can cause autoimmunity.

In this paper, we demonstrate that Roquin<sup>san/san</sup> acts T cell intrinsically to drive an accumulation of T<sub>FH</sub> cells that support a spontaneous GC response. Overexpression of ICOS is a plausible mechanism, because ICOS is important for the generation and survival of T<sub>FH</sub> cells (34, 42, 43), and Roquin normally acts to repress Ies miRNA (28). Previously, we have shown that this accounts for the lymphoproliferation, splenomegaly, and lymphadenopathy of Roquin<sup>san/san</sup> mice (28). The data presented in this paper also show that CD28 signaling is a key contributor to the development of systemic autoimmunity in Roquin<sup>san/san</sup> mice. CD28 is not only expressed on CD4<sup>+</sup> cells (46) and plasma cells (47), and this expression can influence B cell development and antibody responses. Nevertheless, our previous data showing that the increased plasma cell and GC...
numbers, and B cell activation in sanroque are predominantly B cell extrinsic (22), together with the potent effects of SAP deficiency in the sanroque autoimmune phenotype, suggest that CD28 deficiency in T cells rather than in B cells or stromal cells is responsible for the observed effect. Thus, we infer that the sanroque lupus phenotype is T cell mediated. CD28 deficiency considerably decreased the abnormally high levels of ICOS normally found on Roquin<sup>san/san</sup> T cells. This is likely to contribute...
Causes a lymphoproliferative syndrome, danger signals may also be required for the production of autoantibodies that cause end-organ damage. This is because ligands for CD28 are dependent on the abrogation of the lupus phenotype in a manner comparable to halving the gene dose of Icos (28). A nonmutually exclusive explanation is that although ICOS overexpression causes a lymphoproliferative syndrome, danger signals may also be required for the production of autoantibodies that cause end-organ damage. This is because ligands for CD28 are dependent on the abrogation of the lupus phenotype in a manner comparable to halving the gene dose of Icos (28). A nonmutually exclusive explanation is that although ICOS overexpression

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**Figure 4.** Th1 and Th2 cells are present in Roquin san/san mice in the absence of SAP and Tfh cells, but not non-Tfh effector cells, induce a GC response in wild-type mice. (a) Representative flow cytometric contour plots and (b) graphical analysis of GATA3⁺CD44⁺⁺CD4⁺ and Tbet⁺CD44⁺⁺CD4⁺ cells in mice of the indicated genotypes. Data are representative of two independent experiments (n ≥ 4 per group). (c) Representative dot plots of lymph node CD4⁺PD-1⁺⁺CXCR5⁺ (left), Tbet⁺CD44⁺⁺CD4⁺ (middle), and GATA3⁺CD44⁺⁺CD4⁺ (right) cells. (d) Experimental outline for adoptive transfer of Roquin san/san CD4⁺ CD45.2 CD44⁺⁺CD4⁺⁻ CXCR5⁺ or CD4⁺ CD45.2 CD44⁺⁺PD-1⁺⁻ CXCR5⁺ T cells into CD45.1 C57BL/6 mice. (e) Flow cytometric contour plots and (f) dot plots of B220⁺ GL-7⁺ CD95⁺ GC B cells from CD45.1 C57BL/6 recipients 3 wk after adoptive transfer of the indicated cell type. Data were generated from three mice per group (**, P > 0.001). In a, d, and e, the numbers in the plots represent percentages.
on Toll-like receptor ligation, which typically occurs during infections and tissue damage, and potentially after apoptosis. Because mice in this study were housed under specific pathogen-free conditions, one explanation for the source of the danger signal in the san/san model might be the abundance of apoptotic cells in GCs, which overloads the normal nonimmunogenic modes of disposal (11). Apoptotic cells display self-antigens that are the target of the autoimmune response in lupus, and are ligands for TLR7 and/or TLR9 on antigen-presenting cells and/or B cells (48).

Our data indicate that the GC is central to the autoimmune phenotype of Roquin<sup>−/−</sup> mice, because reduction of spontaneous GCs, conferred by halving the gene dose of the transcriptional regulator Bcl6, results in a proportionate amelioration of autoimmune pathology. This heterozygote phenotype is a novel finding. Although previous reports describe intact antibody responses in Bcl6<sup>+/-</sup> mice on day 11 after immunization (26), these are likely to derive predominantly from the extrafollicular response at this early time point. The heterozygote phenotype is consistent with the observation that GC differentiation is accompanied by only a twofold increase in Bcl6 miRNA expression (49). Although our work does not exclude an effect of halving the gene dose of Bcl6 on T<sub>FH</sub> cell formation, it does strongly suggest that the GC reduction seen in Bcl6<sup>+/-</sup> mice is a consequence of reduced B cell–expressed BCL6: in 50% Bcl6<sup>+/-</sup>/50% Bcl6<sup>-/-</sup> mixed bone marrow chimeras, GC B cells were only decreased in GC B cells deriving from Bcl6<sup>-/-</sup> bone marrow. Furthermore, transfer of Bcl6<sup>+/+</sup> SW<sub>Wt</sub> B cells into Bcl6<sup>−/−</sup> recipient mice, providing the major source of T<sub>FH</sub> cells, resulted in normal donor-derived GC numbers after HEL-SRBC immunization.

SAP deficiency abrogates autoimmune in Roquin<sup>−/−</sup> mice. SAP deficiency affects several T cell subsets and natural killer cells (50, 51) but profoundly impairs the ability of T cells to provide help to B cells for GC formation (37–39). Our results demonstrate that SAP deficiency specifically reduces the development of CD4<sup>+</sup> T cells with a T<sub>FH</sub> phenotype, leaving Th1 and Th2 cell formation largely intact. This, together with the evidence presented for the GC dependence of autoimmune, as well as the observation that transfer of Roquin<sup>−/−</sup>/50% T<sub>FH</sub> cells induces spontaneous GC, places aberrant expansion of T<sub>FH</sub> cells at the root of spontaneous GC formation and autoimmunity in Roquin<sup>−/−</sup> mice. This mechanism is consistent with another lupus model, B6.Sle1<sup>−/−</sup>, in which T cells have a transcriptional profile typical of T<sub>FH</sub> cells (21).

SAP deficiency has been shown to ameliorate autoimmunity in another lupus-prone strain, MRL<sup>+/+</sup> mice (52). These mice have been shown to also develop spontaneous GCs (9), although SHM of autoreactive B blasts has been shown to occur in the T zone areas rather than within GCs (16). SAP deficiency has been shown to eliminate the pathogenic CD4<sup>+</sup>CD8<sup>−</sup> T cells that account for the lymphadenopathy of MRL<sup>+/+</sup> mice (52), and there is evidence that autoimmunity in MRL<sup>+/+</sup> mice is T cell dependent (53). It will be interesting to examine whether dysregulated ectopically located T<sub>FH</sub> cells or an extrafollicular counterpart is contributing to autoimmunity in these mice. Finally, although sanroque SAP-deficient mice do not form spontaneous GCs, they still show hypergamaglobulinemia and very mild cell infiltrates in the kidney, suggesting that a non-T<sub>FH</sub>-mediated pathway contributes to these manifestations in Roquin<sup>−/−</sup>/san mice.

Our data add to previous studies demonstrating that SAP deficiency acts B cell extrinsically, causing a profound and selective impairment of GC reactions (37) with little effect on extrafollicular plasma cell generation. The requirement for SAP for effective T<sub>FH</sub> cell function has previously been ascribed to dysregulated kinetics of ICOS up-regulation and increased expression of CD40L (37), which we confirmed in the present study. Our work also shows that SAP signaling is required to maintain elevated baseline levels of ICOS in naive sanroque T cells.

An intriguing finding of this study was the lack of any role for IL-21 in Roquin<sup>−/−</sup>-induced lupus, T<sub>FH</sub> cell accumulation, and spontaneous GC formation. Although recent reports have shown that IL-21 is involved in T<sub>FH</sub> cell generation and optimal GC responses (33, 34), other groups have previously reported normal GC formation with an accumulation in IgG memory B cells in IL21<sup>−/−</sup> mice (54). A recent paper has also shown that IL-21 can be produced by extrafollicular T cells, which contribute to autoimmunity in MRL<sup>+/+</sup> mice (55). Furthermore, IL-21 has been shown to potently induce plasma cell generation from naive B cells (56), suggesting an important role for IL-21 in extrafollicular B cell responses. Even if IL-21 turns out to be critical for GC B cell selection, it is possible that other molecules overexpressed by sanroque T<sub>FH</sub> cells, such as ICOS, substitute for the effects normally mediated by IL-21.

The pathway to systemic autoimmunity identified in this paper highlights the importance of negative selection of self-reactive lymphocytes in GCs. Autoreactive B cells are a normal component of the naive peripheral B cell repertoire. In lupus patients, these cells enter GCs, whereas in healthy individuals, they are excluded (57). Other evidence indicates that in lupus, GCs can generate B cells that have antinuclear specificities from nonautoreactive precursors (7). Furthermore, GCs are increased in lupus (58). Regardless of their ontogeny, autoreactive centrocytes would normally fail to receive selection signals by T<sub>FH</sub> cells, such as ICOS, substitute for the effects normally mediated by IL-21.

According to the prevailing model for T<sub>FH</sub> selection of centrocytes, large numbers of GC B cells compete with each other for the limiting available T cell help (60) and for antigen on follicular dendritic cells. Centrocytes that have acquired higher affinity for antigen would scavenge more antigen from follicular dendritic cells, which would confer on them an avidity advantage in interactions with T<sub>FH</sub> cells compared with lower affinity B cells. Based on this model, we speculate that in Roquin<sup>−/−</sup>/san mice, competition by B cells for T cell help is reduced by the expansion of T<sub>FH</sub> cells. Interactions between centrocytes and T<sub>FH</sub> cells are determined by affinity for MHC–peptide for the TCR, modified by the action of accessory molecules. Loss of SAP in Roquin<sup>−/−</sup>/san mice causes a reduction in ICOS overexpression, potentially increasing the
antigen affinity required to reach the $T_{FH}$ activation threshold in $Roquin^{san/san}$ $Sap^{-/-}$ mice.

Although the lupus phenotype of $Roquin^{san/san}$ mice is Mendelian, apart from rare exceptions (61), lupus is a polygenic disorder. Nevertheless, it is plausible that one or more polymorphisms or mutations that affect $T_{FH}$ homeostasis, such as those that regulate IL-21, ICOS, or ICOSL expression, could contribute to lupus pathogenesis. Because $T_{FH}$ cells are present in peripheral blood, this is a testable hypothesis. Indeed, there is evidence that SLE patients have increased numbers of circulating CD4$^+$ICOS$^+$ cells in their peripheral blood compared with nonautoimmune individuals (62). Because $T_{FH}$ cells are

**Figure 5.** Serum autoantibodies and renal pathology in $Roquin^{san/san}$ $Sap^{-/-}$ mice and reduction of the GC response in SAP-deficient $Roquin^{san/san}$ mice is caused by B cell–extrinsic factors. (a, left) Representative staining of Hep-2 slides for detection of IgG ANA in the serum of 8-wk-old female $Roquin^{san/san}$ and $Roquin^{san/san}$ $Sap^{-/-}$ mice ($n = 5$ per group). (right) Detection of IgG anti-dsDNA serum antibodies in 6-mo-old female $Roquin^{+/+}$, $Roquin^{san/san}$, and $Roquin^{san/san}$ $Sap^{-/-}$ mice determined by immunofluorescence staining using $C. luciliae$ substrate. Data are representative of three independent experiments ($n \geq 5$ per group). (b) Representative images of kidney sections stained with H&E (top) or viewed under an electron microscope (bottom) from 6-mo-old female $Roquin^{san/san}$ $Sap^{-/-}$ mice. $Roquin^{san/san}$ $Sap^{-/-}$ mice show slight mesangial expansion on H&E staining but no electron-dense deposits. Bars: (top) 100 μm; (bottom) 5 μm. (c) Score of nephritis severity in 6-mo-old female $Roquin^{+/+}$, $Roquin^{san/san}$, and $Roquin^{san/san}$ $Sap^{-/-}$ mice as determined by histological analysis according to the criteria given in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20081886/DC1). Each symbol represents one mouse. Horizontal bars indicate medians. (d) Gating strategy for assessing the HEL-specific GC response (top) and graphic representation (bottom) of the total number of HEL-specific GC cells per spleen in mice with the indicated genotypes 7 d after cotransfer of SW HEL B cells and HEL$^{2x}$-conjugated SRBCs. Data are representative of three independent experiments ($n \geq 4$). Each symbol represents one mouse. (e) Gating strategy for determining HEL-specific extrafollicular plasma cells (top) and graphical representation (bottom) of the number of HEL-specific plasma cells 5 d after cotransfer into mice with the indicated genotypes. Data are representative of two independent experiments ($n \geq 3$ per group). p-values are indicated on graphs.
the cells expressing the highest levels of ICOS (63, 64), it is possible that circulating CD4\(^+\)ICOS\(^+\) cells reflect an excessive T\(_{FH}\) response, and is consistent with recent data showing that a subset of SLE patients have an increased proportion of CD4\(^+\)C D45RO\(^-\)CXCR5\(^+\)ICOS\(^{high}\)PD-1\(^{high}\) cells in their peripheral blood (a phenotype that correlates with higher titers of anti-dsDNA antibodies and more severe kidney damage; unpublished data). If confirmed in independent studies, the GC–T\(_{FH}\) pathway elucidated in the Roquin\(^{Rosa} /^{Rosa}\) model pathway would emerge as a novel and specific target for therapy.

Figure 6. T\(_{FH}\) cell–associated molecules are decreased in Roquin\(^{Rosa} /^{Rosa}\) Sap\(^{-/}\) mice. (a) Representative flow cytometric histograms and (b and c) graphical analysis showing ICOS mean fluorescence intensity (MFI) of splenic naive CD44\(^{-}\) (b) and activated/memory CD44\(^{+}\) (c) CD4\(^+\) T cells from 10-wk-old unimmunized mice of the indicated genotypes. Data are representative of three independent experiments. Each symbol represents one mouse. (d) ELISA quantification of IL-21 in supernatant from an overnight culture of splenocytes in the presence of PMA and ionomycin from mice of the indicated genotypes. Data are representative of three experiments. (e) Flow cytometric contour plots of CD40L expression on splenocytes, 5 h after stimulation with anti-CD3 and anti-CD28, derived from mice of the indicated genotypes. (left) Staining with an isotype control; (right) CD40L staining. (f) Histograms of the percentage of CD4\(^+\) cells that express CD40L (gated as shown in e) 5 h after CD3 and CD28 stimulation on splenocytes from mice with the indicated genotypes. In d and f, error bars indicate means ± SEM.
housed in specific pathogen-free conditions at the Australian National University Bioscience Facility. IL-21 KO mice were generated at Lexicon Pharmaceutics, Inc. and were provided by M. Smyth (Peter MacCallum Cancer Center, Melbourne, Australia). Mice and immunizations. Roquin<sup>San/san</sup> and C57BL/6 (B6) mice, all crossed to Sap<sup>-/-</sup>, Bcl6<sup>+/+</sup>, Cd28<sup>-/-</sup>, and IL-21<sup>-/-</sup> mice, and SWHEL mice, were

**Figure 7.** Lack of IL-21 does not affect the phenotype, T<sub>FH</sub> cell accumulation, or GC formation of Roquin<sup>San/san</sup> mice. (a) IgG ANAs in the serum of mice of the genotypes indicated, detected by immunofluorescence using Hep-2 substrate. Data are representative of three independent experiments (n ≥ 5 mice per group). (b) Score of ANA staining intensity by confocal microscopy from sera taken from mice of the indicated genotypes. Data are representative of two independent experiments (n ≥ 5 mice per group). (c) Basal serum total IgG, IgG1, and IgE measured by ELISA. Data are representative of two independent experiments (n = 5 mice per group). (d) Lymph node and spleen weight in grams for mice of the indicated genotypes. Data are representative of two independent experiments (n ≥ 5 per group). (e) Flow cytometric contour plots and dot plots of PD-1<sup>high</sup>CXCR5<sup>+</sup>CD4<sup>+</sup> T<sub>FH</sub> cells and (f) GL-7<sup>+</sup>CD95<sup>+</sup>B220<sup>+</sup> GC cells from mice of the indicated genotypes. Data are representative of two independent experiments (n ≥ 5 mice per group). In e and f, the numbers in the plots represent percentages.
Centre, Melbourne, Australia) and ZymoGenetics, Inc. These mice were backcrossed 10 generations onto the C57BL/6 background. SWI/h mice carry a Vhi-κ light chain transgene and a knocked in VH10 Ig heavy chain in place of the JH segments of the endogenous IgH gene that encode a high-affinity antibody for HEL (65). All animal procedures were approved by the Australian National University Animal Ethics and Experimentation Committee.

To generate thymus-dependent responses where indicated in the figures, 8–12-wk-old mice were immunized i.p. with 2 × 10^7 SRBCs (Institute of Medical and Veterinary Science Veterinary Services). For experiments involving SWI/h mice, 10^6 SWI/h B cells were transferred into recipients, which were simultaneously immunized i.v. with 2 × 10^7 SRBCs conjugated with mutant HEL<sup>+</sup> using a protein conjugation kit (Invitrogen) (41).

### Bone marrow chimeras
Recipient C57BL/6-Ly5a mice were sublethally irradiated with 1,000 rad and were reconstituted via i.v. injection with 2 × 10^6 donor bone marrow–derived hematopoetic stem cells.

### Antibodies
Antibodies and streptavidin conjugates for flow cytometry were from BD unless otherwise indicated: anti-mouse B220-PerCP, CD4-PerCP, ICOS-PE (eBioscience), FoxP3 (eBioscience), GL-7–FITC, GATA3–allophycocyanin, Tbet–PerCP Cy5.5, CD95–PE, CXCR5–biotin, PD-1–PE (eBioscience), CTLA-4–PE, CD25–allophycocyanin, streptavidin–PerCP Cy5.5, and CD40L–biotin. For immunohistochemistry, the primary antibodies and reagents used were rat anti–mouse IgD (SouthernBiotech), biotinylated anti–mouse TCR β (BD), rat anti–mouse PD-1 (BioLegend), and PNA–biotin (Vector Laboratories); the secondary antibody used was rabbit anti–rat horseradish peroxidase (HRP; Dako).

### Cell isolation, culture, and stimulation
Single-cell suspensions were prepared from spleens of unimmunized and/or immunized mice. Single-cell suspensions were prepared in RPMI 1640 medium (JRH Biologies) supplemented with 2 mM l-Glutamine (Invitrogen), 100 U penicillin–streptomycin (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 100 mM Hepes (Sigma–Aldrich), 5 × 10<sup>−5</sup> 2-mercaptoethanol, and 10% fetal calf serum by sieving and gentle pipetting through 70-μm nylon mesh filters (Falcon; BD). Cells were then cultured for 24 h at 37°C/5% CO<sub>2</sub>, with 50 ng/ml PMA (Sigma–Aldrich) and 1 μM monomycin (Sigma–Aldrich) in triplicate wells. Cell culture supernatants were aspirated for further analysis by ELISA. For CD40L expression, cells were cultured in triplicate wells for 5 h in 96-well plates (Costar; Corning) with platebound 1 μg/ml anti-CD3 (BD) and 10 μg/ml anti-CD28 (BD). Biotinylated anti-CD40L antibody was added 2 h before harvesting.

### Flow cytometry
For surface staining, single-cell suspensions were prepared as described in the previous section, and cells were maintained in the dark at 4°C throughout. Cells were washed twice in ice-cold FACS buffer (2% fetal calf serum, 0.1% NaN<sub>3</sub> in PBS), and incubated with each antibody and conjugate layer for 30 min and washed thoroughly with FACS buffer between each layer. Intracellular staining was performed using the Cytofix/Cytoperm kit (BD) according to the manufacturer’s instructions. For detection of HEL–binding B cells, HEL was conjugated to Alexa Fluor 647 using the Cytofix/Cytoperm kit (BD) according to the manufacturer’s instructions. For detection of HEL–binding B cells, HEL was conjugated to Alexa Fluor 647 using the Cytofix/Cytoperm kit (BD) according to the manufacturer’s instructions.

### Adoptive cell transfer experiments
T<sub>eff</sub> cells (CD<sup>4</sup>CD<sup>4</sup>–FITC–PE–HCDC–CXCR3<sup>+</sup>) and non-T<sub>eff</sub> effector cells (CD<sup>4</sup>CD<sup>4</sup>–FITC–PE–HCDC–CXCR3<sup>+</sup>) from spleens and lymph nodes were prepared and stained as described in the previous paragraph and sorted on a FACSAnia (BD). 5 × 10<sup>6</sup> sorted cells were resuspended in PBS and injected into the tail vein of unmanipulated CD45.1 C57BL/6 recipients. 3-wk after transfer, splenic donor (CD45.2) T<sub>eff</sub> cells and host CD<sub>4</sub> cells were enumerated by flow cytometry.

ELISA. Maxisorb plates (Thermo Fisher Scientific) were coated with 100 μg/ml of recombinant mouse IL–21R–Fc (R&D Systems). Serial supernatant dilutions were applied in quadruplicate, and IL–21 concentration was determined with 200 ng/ml of HRP–conjugated goat anti–mouse IL–21 IgG (R & D Systems). Bound IL–21 was detected using phosphatase substrate tablets (Sigma–Aldrich). Plates were read at 405 nm using a microplate reader (Thermomax; MDS Analytical Technologies). The titers for supernatant samples were calculated according to the standard curve generated using twofold dilutions of recombinant mouse IL–21 (500–15 pg/ml; R&D Systems) and Pristin software (GraphPad Software, Inc.). IL–17 ELISA was performed using the mouse IL–17A ELISA Ready–Set–Go! Kit (eBioscience) according to the manufacturer’s instructions. For analysis of total IgG, IgG1, and IgE titers, Maxisorb plates were coated with goat anti–mouse κ light chain or anti–mouse IgE (BD). Serial serum dilutions were applied, and Ig concentration was determined with HRP–conjugated goat anti–mouse IgG, IgG1 (SouthernBiotech), or biotinylated IgE (BD), followed by streptavidin–HRP. The enzyme bound to plates was developed using phosphatase substrate tablets. Plates were read at 405 nm using a Thermomax microplate reader. The titers for serum samples were calculated as the log serum concentration required to achieve 50% maximum OD.

### Immunohistochemistry
5-μm acetone–fixed frozen sections of spleen were air dried and washed in 0.1 M Tris-buffered saline (TBS), pH 7.6, and were stained with various antibodies for 45 min at room temperature in a moist chamber. After a further wash in TBS, secondary reagents, previously absorbed in 10% normal mouse serum, were added to the sections for 45 min. Where biotin–conjugated primary or secondary reagents were used, streptavidin–alkaline phosphatase (Vector Laboratories) was added after a further wash in TBS and incubated for 20 min. HRP activity was detected using diaminobenzidine tetrahydrochloride solution (Sigma–Aldrich) and hydrogen peroxide. Alkaline phosphatase activity was detected using the AP Substrate Kit III (Vector Laboratories). Sections were mounted with IMMUT–MOUNT (Thermo Shandon) and viewed under a microscope (model IX71; Olympus).

### Renal pathology
Kidneys were fixed in 10% neutral buffered formalin, 4-μm paraffin–embedded sections were dewaxed and stained with hematoxylin and eosin (H&E) and silver stains. For electron microscopy, kidneys were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature overnight. They were then washed in 0.1 M cacodylate buffer, treated with 0.2 mm of filtered 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 2 h, and washed in double–distilled H<sub>2</sub>O. Samples were stained with 0.2 mm of filtered 2% aqueous uranyl acetate for 30 min at room temperature, dehydrated in ethanol, and embedded in 100% TAAB medium mix, low viscosity resin. They were then mounted in orientation silicon moulds and heated in an oven at 70°C for 8–12 h. The samples were examined on a transmission electron microscope (model 1011; JEOL) at 60 kV. The images were captured using a digital camera (MegaView III) and the AnalySIS software package (Soft Imaging System; Olympus), and were subsequently scored using the criteria detailed in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20081886/DC1).

### ANA and dsDNA assessment
Serum obtained by eye bleed from 8-wk-old mice was diluted in PBS 1:40 and used for indirect immunofluorescence on fixed Hep–2 slides (Antibodies, Inc.) for ANA detection, and serum from 6–mo-old mice was diluted 1:20 and used for indirect immunofluorescence on fixed C. fasciatae slides (Antibodies, Inc.) for ANA anti–dsDNA antibody detection, respectively. Alexa Fluor 488 goat anti–mouse IgG (Invitrogen) was used to detect mouse antibodies. Autoantibodies were scored blind as negative or positive on a scale of 1–3 based on the intensity of fluorescence. Relative levels of ANAs were estimated by viewing the slides using a confocal microscope (TCS SP5; Leica) at 20× magnification and a fixed laser power, and measuring the fluorescence of five randomly selected 1,250-μm<sup>2</sup> regions of Hep2 cells, compared with five regions where cells were absent. The sample–specific mean background was subtracted from the sample–specific mean fluorescence to give an estimation of fluorescent intensity. These results were subjected to a blinded manual scoring of fluorescence for the same samples.

### Statistical analysis
Data were analyzed using a two–tailed Student’s t test using Prism software.
Online supplemental material. Fig. S1 illustrates that the loss of one allele of Bcl6 results in B cell–intrinsic defects in GC formation. Fig. S2 a shows that CD40 Foxp3+ T reg cells are expanded twofold in Roquin−/− mice relative to Roquin+/+ mice. Fig. S2 b demonstrates that Roquin+/− splenocytes produce equivalent levels of IL-17 to Roquin+/+ splenocytes. Fig. S3 (a and b) shows that Sap deficiency does not correct the splenomegaly and lymphadenopathy in Roquin−/− mice. Fig. S3 c demonstrates that hyper-IgG in Roquin+/− mice is not corrected in the absence of Sap. Table S1 describes the scoring strategy used to assess the severity of mouse nephritis. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081886/DC1.

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Figure S1. Heterozygosity for Bcl6 within the B cell compartment reduces the magnitude of the GC response. (a) Dot plots of B220⁺GL-7⁺CD95⁺ GC B cells from mixed bone marrow chimeras 8 d after SRBC immunization. Chimeric mice were generated by sublethal irradiation of recipient C57BL/6-Ly5a mice and reconstitution with a 1:1 mix of either Bcl6⁺/+Ly5a/Bcl6⁺/+ or Bcl6⁺/-Ly5b/Bcl6⁺/-Ly5b bone marrow. Immunizations were performed 12 wk after reconstitution. Data are representative of two independent experiments (n = 4 per group). (b) Experimental outline for evaluating the HEL-specific GC cell response after co transfer of SWHEL B cells and HEL-2x-conjugated SRBC. (c) Gating strategy for assessing the HEL-specific GC response. (d) Dot plots showing the total number of CD45.1 donor derived HEL-binding GL-7⁺CD95⁺ B220⁺ GC B cells 8 d post intravenous transfer of 10⁴ SWHEL B cells and SRBC conjugated to HEL₂x into 10-wk-old recipient mice of the genotypes indicated. Data are representative of 4 independent experiments (n = 4 per group). In a and d each symbol represents one mouse and P values are indicated on the graphs.
Figure S2. T reg cell number and IL-17 production are not reduced by the san allele of Roquin. (a) Representative flow cytometric contour plots (left) and graphical representation (right) of CD4+CD25+FoxP3+ T reg cells in 10-wk-old Roquin<sup>san/san</sup> mice compared with littermate controls (Roquin<sup>+/+</sup>). Each symbol represents one mouse. Data are representative of five independent experiments. The numbers in the plots represent percentages, and horizontal bars indicate medians. (b) ELISA was used to determine culture supernatant IL-17 levels from 24-h splenocyte cultures from Roquin<sup>san/san</sup> mice and littermate controls in the presence (shaded bars) or absence (open bars) of PMA and ionomycin. Data are representative of three independent tests. Error bars indicate means ± SEM.
Figure S3. SAP deficiency does not correct the splenomegaly or lymphadenopathy seen in Roquin$^{san/san}$ mice. (a) Photographs of the spleen and lymph nodes (cervical, inguinal, axillary, subscapular, paraaortic, and mesenteric) from 10-wk-old Roquin$^{san/san}$ and Roquin$^{san/san}$ Sap$^{+/+}$ mice. (b) Spleen weights and (c) weights of pooled lymph nodes (cervical, inguinal, axillary, subscapular, paraaortic, and mesenteric) from 10-wk-old Roquin$^{san/san}$ and Roquin$^{san/san}$ Sap$^{+/+}$ mice. Differences are not statistically significant. (d) ELISA analysis of total serum IgG from unimmunized 10-wk-old mice of the indicated genotypes. Data are representative of three experiments. Horizontal bars indicate medians.
Table S1. Scoring strategy used to assess the severity of mouse nephritis

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<td>Proliferative or fibrinoid GN without crescents</td>
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<td>GN with fibrinoid or crescents in &lt;50% of glomeruli</td>
<td>Moderate scarring</td>
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<tr>
<td>4</td>
<td>GN with fibrinoid or crescents in &gt;50% of glomeruli</td>
<td>Fibrous obliteration</td>
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NAD, no abnormality detected.