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Circadian clock cryptochrome proteins regulate autoimmunity

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The circadian system regulates numerous physiological processes including immune responses. Here, we show that mice deficient of the circadian clock genes *Cry1* and *Cry2* [*Cry* double knockout (DKO)] develop an autoimmune phenotype including high serum IgG concentrations, serum antinuclear antibodies, and precipitation of IgG, IgM, and complement 3 in glomeruli and massive infiltration of leukocytes into the lungs and kidneys. Flow cytometry of lymphoid organs revealed decreased pre-B cell numbers and a higher percentage of mature recirculating B cells in the bone marrow, as well as increased numbers of B2 B cells in the peritoneal cavity of *Cry* DKO mice. The B cell receptor (BCR) proximal signaling pathway plays a critical role in autoimmunity regulation. Activation of *Cry* DKO splenic B cells elicited markedly enhanced tyrosine phosphorylation of cellular proteins compared with cells from control mice, suggesting that overactivation of the BCR-signaling pathway may contribute to the autoimmunity phenotype in the *Cry* DKO mice. In addition, the expression of *C1q*, the deficiency of which contributes to the pathogenesis of systemic lupus erythematosus, was significantly down-regulated in *Cry* DKO B cells. Our results suggest that B cell development, the BCR-signaling pathway, and *C1q* expression are regulated by circadian clock CRY proteins and that their dysregulation through loss of CRY contributes to autoimmunity.

cryptochrome | autoimmune | B cell receptor

Circadian clocks drive rhythms in physiology and behavior enabling organisms to keep track of the time of day and to help anticipate and adapt to recurrent and predictable daily changes in the environment (1). In mammals, the circadian timing system has a hierarchical architecture, in which the hypothalamic suprachiasmatic nucleus (SCN) functions as a light-responsive central clock generating neural and hormonal signals to peripheral clocks that are present in virtually all cells of the body (2). At the molecular level, mammalian circadian clocks in the SCN are controlled by transcriptional and translational feedback loops. A heterodimeric protein complex of "circadian locomotor output cycles kaput" (CLOCK) and "brain and muscle ARNT-like 1" (BMAL1) drives transcription through E-box elements in promoters of target genes, including their own repressors, Period (*Per1*, *Per2*, and *Per3*) and Cryptochrome (*Cry1* and *Cry2*), which in turn repress BMAL1 and CLOCK activity (3). "Reverse orientation c-erb" (REV-ERB) and "RAR-related orphan receptor" (ROR) also participate in the rhythmic transcriptional activity of the molecular oscillator (4). Similar to the SCN, the molecular clockwork in peripheral cells is composed of autoregulatory transcription-translation feedback loops orchestrated by the circadian clock genes (5). The peripheral oscillators, synchronized by the central clock, control the expression of downstream clock-controlled genes in a tissue-specific manner (6).

Circadian rhythms have long been known to play crucial roles in physiology. More and more reports suggest that they act as important regulators of specific immune functions (7, 8). For example, Toll-like receptor 9 (TLR9), a member of the toll-like receptor family that plays a fundamental role in pathogen recognition and activation of innate immunity, is regulated by BMAL1/CLOCK (9). Another example is Interleukin-17–producing $CD4^+$ T helper (T_H17) cells, which are proinflammatory immune cell guardians against bacterial and fungal infections at mucosal surfaces. The differentiation of these cells varies diurnally and is altered in *Rev-erbα*$^{-/-}$ mice (10). Furthermore, macrophages from murine spleens, lymph nodes, and peritoneum produce different levels of

Significance

Accumulated data show that circadian rhythms act as important regulators of specific immune functions, including activities of Toll-like receptor 9, macrophages, and Interleukin-17–producing $CD4^+$ T helper (T_H17) cells. In this study, we show that mice deficient of the circadian clock genes *Cry1* and *Cry2* unexpectedly display an autoimmune phenotype including high serum IgG concentrations, the presence of serum antinuclear antibodies, and precipitation of IgG, IgM, and complement 3 in glomeruli. Our results suggest that B cell development, the B cell-receptor–signaling pathway, and *C1q* expression are regulated by CRY proteins and that their dysregulation contributes to autoimmunity.

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TNF-α and IL-6 when stimulated by bacterial endotoxin at different times during the circadian cycles, indicating that the intrinsic circadian clock regulates inflammatory innate immune functions (11). The circadian oscillations of immune mediators coinciding with the activity of the immune system may help to promote tissue recovery and possibly allow the host to anticipate and more efficiently handle microbial threats (7). Genetic silencing of circadian clock genes has a broad effect on immunity (12).

Molecular clocks have been characterized in B lymphocytes (13). Variations of lymphocyte numbers in peripheral blood have been reported (14). Knocking out the circadian gene *Bmal1* in mice affects B cell development (15), indicating the close interaction between circadian rhythm and B cell regulation. However, the mechanisms relating to how development and function of B cells are affected by circadian rhythm or circadian proteins remain largely unknown. The B cell-receptor (BCR) complex is composed of two parts: (*i*) a membrane-bound Ig-like molecule and (*ii*) a noncovalently associated Ig-α and Ig-β which contain cytoplasmic domain immunoreceptor tyrosine-based activation motifs (ITAMs). BCR functions are required for normal antibody production, and defects in BCR signal transduction may lead to immunodeficency, autoimmunity, and B cell malignancy. Cross-linking of BCR results in tyrosine phosphorylation of the ITAMs by the SRC-family tyrosine kinase LYN followed by recruitment and activation of SYK tyrosine kinase, which in turn phosphorylates key downstream substrates such as BLNK (B cell linker), BTK (Bruton's tyrosine kinase), PLC-γ 2 (phospholipase C-γ2), and Rho-family GTP-GDP exchange factor Vav, resulting in robust BCR signaling as well as activation and productive interactions with helper T cells.

Here, we show that *Cry* double knockout (DKO) mice manifest an autoimmune-like phenotype. The *Cry* deficiencies substantially enhance the rate of B cell maturation, not only affecting early B cell development in the bone marrow (BM) but also stimulating specific B cell developmental subpopulations in the spleen and peritoneal cavity, leading to an increase in serum IgG levels and autoantibody production. Prior studies have shown that free-running rhythm is abolished in mice lacking both *Cry*1 and *Cry*2 (16). Modern life often involves chronic circadian disruptions, such as night shift work or jet lag that are linked to human inflammatory diseases. Our findings suggest that the pathologic consequences of circadian disruption may be due in part to direct interactions between the circadian clock and the pathways that regulate B cell development and tolerance.

Results

***Cry* DKO Mice Spontaneously Manifest Autoimmune-Like Disease.** The deletion of CRY in BM cells from $Cry1^{-/-}Cry2^{-/-}$ (*Cry* DKO) mice (17) was confirmed by Western blot (Fig. 1*A*). *Cry* DKO mice have a substantially (five- to sixfold) higher level of serum IgG antibodies compared with WT C57BL/6J (B6) mice, while the serum levels of IgM were similar between *Cry* DKO and WT mice (Fig. 1*B*). Given this dysregulation, we investigated whether immune tolerance was impaired in the *Cry* DKO mice by examining for the presence of antinuclear antibodies (ANA). Immunofluorescent staining revealed robust ANA in the sera of *Cry* DKO mice compared with WT mice (Fig. 1*C*), suggesting that the *Cry* DKO mice may be more prone to develop autoimmune diseases. Immunohistological analysis of kidney sections of the mutant mice revealed increased glomerular deposits of IgG and IgM antibodies and complement 3 (C3) compared with controls (Fig. 1*D*). Furthermore, electron microscopic examination showed massive immune complex deposits in the mesangium of the kidneys of *Cry* DKO mice (Fig. 1*E*). To assess directly whether *Cry* DKO mice manifest features of autoimmune diseases, histopathological analysis of various tissues from the mutant and control mice was performed. Compared with the lungs and kidneys of WT mice where leukocytes were not detected, massive infiltrations of leukocytes were evident in the *Cry* DKO mice (Fig. 1*F*, *Left* and *Right*, respectively). These results are consistent with the *Cry* DKO mice having an autoimmune disorder. To determine whether loss of *Cry* in hematopoietic cells is sufficient for the autoimmune disorder, donor BM cells from either WT B6 or *Cry* DKO mice were transplanted into lethally irradiated WT B6 or *Rag1* KO mice. As shown in Fig. 1 *G* and *H*, serum IgG and IgM concentrations were significantly elevated in *Cry* DKO BM transplanted mice compared with the control WT B6 BM transplanted mice. Furthermore, increased deposition of IgG/IgM immune complexes was observed in the kidneys of *Cry* DKO chimeras at 2 mo post BM transplantation (Fig. 1*I*).

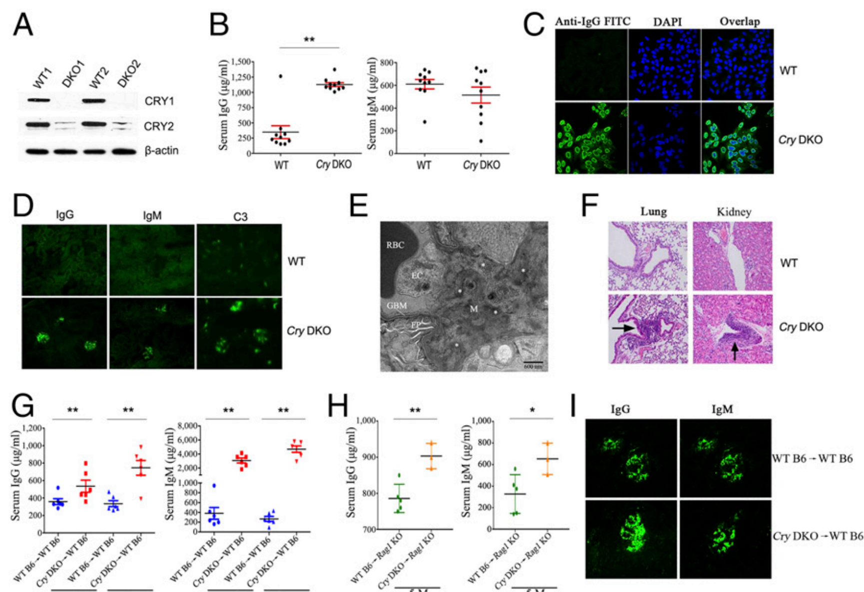

Fig. 1. Serum antibody titers and pathological analyses of *Cry* DKO mice. (*A*) Western blot analysis of CRY1 and CRY2 in BM cells of WT and *Cry* DKO mice. (*B*) Serum concentrations of IgG and IgM. Data were collected from 8- to 10-wk-old WT and *Cry* DKO mice. Each symbol represents data from one individual mouse. $^{**}P < 0.01$ (two-tailed, unpaired *t* test). (*C*) Serum ANA in *Cry* DKO mice. Sera samples (1:160 dilution) from *Cry* DKO mice and WT littermates were used for the ANA analysis. Shown are Hep2 cells stained with sera from WT and *Cry* DKO mice. Bound IgG (green) was detected with FITC-conjugated anti-mouse IgG. (Magnification, 200×.) (*D*) Ig deposits in kidney of *Cry* DKO mice. Kidney sections from WT and *Cry* DKO mice were immunofluorescently stained with anti-murine IgG (IgG, *Left*), anti-murine IgM (IgM, *Middle*), or anti-murine complement 3 (C3, *Right*). Results are from four representative *Cry* DKO mice and four WT mice. Glomeruli of Cry DKO mice had IgG, IgM, and C3 antibody deposits. Images were taken at 200× magnification. (*E*) Electron microscopy showed immune complexes (*) deposited in the mesangium of the glomeruli of *Cry* DKO mice. EC, endothelial cells; FP, foot process; GBM, glomerular basement membrane; M, mesangium; RBC, red blood cell. (*F*) Perivascular infiltration of leukocytes in *Cry* DKO mice (marked by arrows). Lung and kidney sections from WT and *Cry* DKO mice were stained with hematoxylin and eosin (H&E). (Magnification, 200×.) (*G* and *H*) B6 mice (*G*) or *Rag1* KO mice (*H*) were irradiated and reconstituted with either WT B6 or *Cry* DKO BM. Serum concentrations of IgG and IgM were examined in BM transplanted mice at indicated times post transplantation. $^{*}P < 0.05$; $^{**}P < 0.01$ (two-tailed, unpaired *t* test). (*I*) Ig deposits were more prominent in glomeruli of *Cry* DKO chimeras. B6 mice were irradiated and reconstituted with either WT B6 or *Cry* DKO BM. Kidney sections from BM transplanted mice were stained with immunofluorescent anti-murine IgG (IgG, *Left*) and anti-murine IgM (IgM, *Right*). Results are from six representative BM transplanted mice in each group as indicated. (Magnification, 400×.)

Protein arrays were used to characterize a wide spectrum of autoantibodies to identify autoantibody clusters that best predict autoimmune disease activity. Newer approaches permit measurement of dozens of autoantibodies simultaneously using small volumes of serum. These protein arrays provide greater sensitivity and dynamic range than ELISA (18). An autoantigen microarray panel was used—which contained 95 autoantigens and eight internal control antigens, covering autoantigens present in patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), Sjögren's syndrome, inflammatory muscle diseases, and other autoimmune disorders—to examine sera samples from WT B6 versus *Cry* DKO mice and WT B6 BM transplanted versus *Cry* DKO BM transplanted mice. The sera from *Cry* DKO mice and *Cry* chimeras reacted strongly to most glomerular and nuclear antigens, suggesting that loss of CRY proteins leads to autoimmunity in vivo (Fig. 2).

Differential Alterations of BCR-Proximal Signaling in *Cry* DKO cells. Gene expression was analyzed by microarrays to identify genes that are differentially regulated in *Cry* WT and DKO BM cells. Genes were sorted based on the ratio of gene expression values (Fig. 3*A*). *Cry* DKO cells showed a disproportionate up-regulation of recombinant Ig heavy and light chain genes. Gene set enrichment assay (GSEA) of expression array data from WT and *Cry* DKO BM cells showed up-regulation of the BCR pathway in *Cry* DKO cells (Fig. 3*B*). Vκ-Jκ and Vλ-Jλ gene rearrangements occur in B cell development in vivo and in vitro (20, 21). Withdrawal of IL-7 induced the expected transition from large, cycling pre-B cells to small, resting pre-B cells and the subsequent expression of κ light chains on the cell surface (Fig. 3*C*). Notably, the percentage of cells with κ light chain expression was markedly increased in *Cry* DKO compared with WT cells (Fig. 3 *C* and *D*). Likewise, *Cry* DKO spleen B cells elicited enhanced BCR-proximal signaling after anti-IgM stimulation, as manifested by tyrosine phosphorylation of cellular proteins compared with WT B cells (Fig. 3*E*). In particular, total VAV levels, as well as the phosphorylation levels of BTK, Igα, SRC, and PLC-γ 2, were increased in *Cry* DKO B cells compared with control cells (Fig. 3*F*). Taken together, these results show that loss of CRY proteins affects the strength of multiple BCR-proximal signal-ing pathways.

Altered B cell Development in *Cry* DKO Mice. To determine whether loss of CRY altered B cell development, the B cell compartments of the BM, spleen, and peritoneal cavity were analyzed by flow cytometry (Fig. 4). No differences in the numbers of Lin-, Sca1+, c-kit- (LSK) cells and common lymphoid progenitor cells (CLP) were noted in the BM of *Cry* DKO and WT mice (Fig. S1). In contrast, BM mature B cells and peritoneal B2 cells were increased significantly in *Cry* DKO mice (Fig. 4 *A*, *B*, *F*, and *G*). Splenic marginal zone B cells were decreased significantly in *Cry* DKO mice (Fig. 4 *C* and *D*), while spleen sizes were equivalent in the WT and *Cry* DKO mice (Fig. 4*E*). Also, the percentage of B cells in peripheral blood was not significantly different between WT and *Cry* DKO mice (Fig. 4*H*). These findings indicate that silencing *Cry* alters the development of multiple B cell subsets in both the BM and the periphery. No significant changes in numbers of regulatory T lymphocytes (Tregs) occurred in the splenic cells of WT and *Cry* DKO mice (Fig. S2).

***Cry* DKO B Cells Have a Hyperactive Response to T-Independent Antigen Stimulation.** To determine whether the silencing of *Cry* affects B cell activation in vivo, the T-dependent (TD) and T-independent (TI) antibody responses were examined. WT and *Cry* DKO mice were immunized either with 4-hydroxy-3-nitrophenyl-acetyl (NP)-conjugated keyhole limpet hemocyanin (KLH) for TD antibody responses or with either NP-Ficoll (type I) or NP-lipopolysacharide (LPS, type II) for TI antibody responses. Sera samples were collected on days 7 and 14 after immunization and subjected to ELISA with specific antibodies (Fig. 5). The NP-specific IgM and IgG2a responses in NP-KLH–immunized *Cry* DKO mice were comparable to WT mice, while the production of NP-specific IgG1 in the mutant mice was

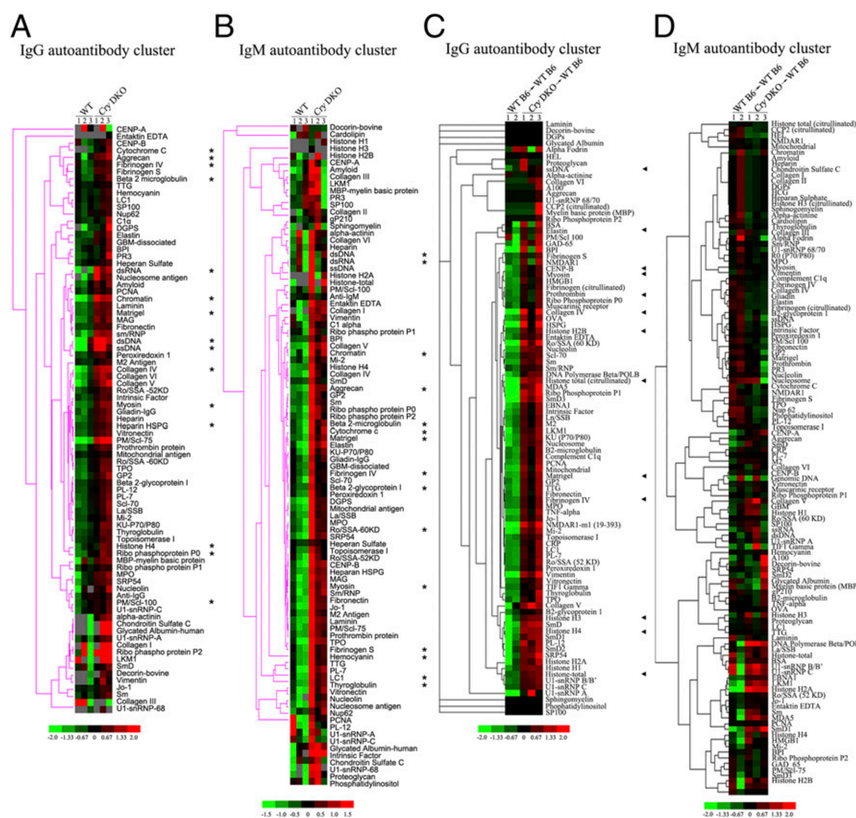

Fig. 2. Heat map with clustering of IgG (*Left*) and IgM (*Right*) autoantibodies in *Cry* DKO mice and *Cry* DKO chimeras. (*A* and *B*) Sera were collected from 10-wk-old WT or *Cry* DKO mice and analyzed for autoantibodies using an auto-antigen microarray as described. (*C* and *D*) B6 mice were lethally irradiated and reconstituted with WT B6 or *Cry* DKO BM. Sera were collected from BM transplanted mice at 2 mo post transplantation and submitted to auto-antigen microarray. Antibody titers are presented as colors comparing *Cry* DKO versus WT B6 mice as well as *Cry* DKO BM transplanted mice versus WT B6 BM transplanted mice. Signal intensities higher and lower than the mean value of the raw data are represented in red and green, respectively. Signals either close or equal to the mean value of the raw data are depicted as gray or black. Autoantibodies with similar up-regulation in different murine lupus strains [New Zealand black (NZB)/NZ white (W) F1, NZ mixed (NZM) 2410, Murphy Roths large (MRL)/lpr, and BXSB] (19) are marked with an asterisk (*) (*A* and *B*) or an arrowhead (◂) (*C* and *D*).

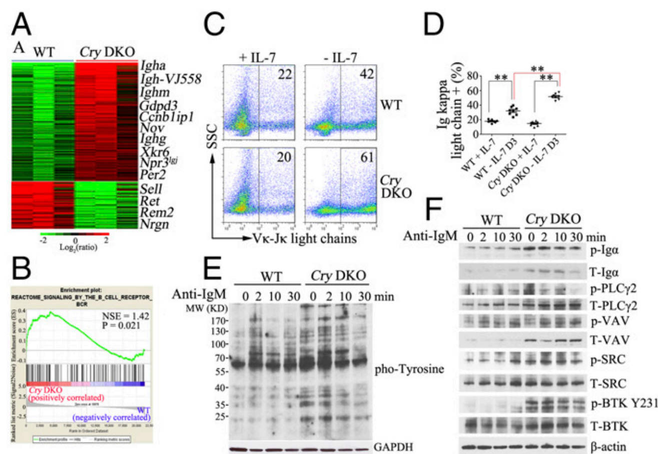

Fig. 3. Dysregulation of BCR downstream signaling pathways in *Cry* DKO B cells. (*A*) BM cells from either 8-wk-old WT or *Cry* DKO mice were RNA-extracted and followed by RNA array analysis. The largest up- and down-regulated genes in *Cry* DKO cells are labeled. (*B*) GSEA showed that the B cell receptor pathway was up-regulated in *Cry* DKO cells. (*C* and *D*) BM cells from eight WT and eight *Cry* DKO were cultured with IL-7 for 1 wk and then withdrawn from the culture medium for 3 d, and FACS analysis for cell-surface Ig kappa light chain was performed. $^{**}P < 0.0001$ (two-way ANOVA). (*E* and *F*) Purified B cells were stimulated with anti-IgM F(ab′)2 for various durations as indicated. Level of expression of tyrosine phospho-proteins (*E*) and BCR pathway proteins (*F*) in the cell lysates was determined by immunoblot analyses with either a phospho-tyrosine antibody (*E*) or the indicated antibody.

moderately lower than that present in control mice (Fig. 5*A*). In contrast, after NP-Ficoll immunization, the *Cry* DKO mice had higher titers of anti-NP IgM and IgG (Fig. 5*B*). Furthermore, the NP-specific IgM and IgG levels were higher in NP-LPS–immunized *Cry* DKO mice than control mice at day 14 after immunization (Fig. 5*C*). These results suggest that the autoimmunity in *Cry* DKO mice is likely due to a generalized B cell hyperactivation.

Down-Regulation of *C1q* in *Cry* DKO Cells. *C1q* is composed of 18 polypeptide chains including six A-chains, six B-chains, and six C-chains. Deficiency of *C1q* has been associated with lupus erythematosus and glomerulonephritis (23, 24). Patients suffering from lupus erythematosus often have deficient expression of *C1q*. RNA-sequencing analysis of CD19+ spleen B cells revealed reduced expression levels of *C1qa*, *C1qb*, *C1qc*, *C6*, *Fcgr3*, and *Fcgr4* in *Cry* DKO cells (Fig. 6*A*), which was confirmed by qPCR (Fig. 6*B*).

Discussion

Our findings reveal that the CRY circadian proteins are involved in autoimmune regulation. Indeed, *Cry* DKO mice develop an autoimmune phenotype as shown by high serum IgG concentrations, the presence of serum antinuclear antibodies, and the precipitation of IgG, IgM, and C3 in their glomeruli (Figs. 1 and 2). Previously, loss of CRY proteins was shown constitutively to increase the expression of the proinflammatory cytokines including IL-6, TNF-α, and iNOS in fibroblasts and bone-marrow–derived macrophages in vitro and to potentiate the immune system and significantly elevate secretion of proinflammatory cytokines upon LPS challenge in vivo (25). Lymphocytes from *Cry* DKO mice also produced excessive TNF-α and heightened inflammatory joint disease with up-regulation of essential mediators of arthritis including TNF-α, IL-1b, IL-6, and matrix metalloproteinase-3 (26).

Cyclic adenosine monophosphate (cAMP) formed from ATP by the action of adenylyl cyclase is an important intracellular second messenger that activates the cAMP-dependent protein kinase A (PKA) and mediates many different cellular responses including regulation of metabolism and gene transcription. cAMP is also a potent regulator of innate and adaptive immune cell functions (27). Narasimamurthy et al. (25) showed that CRY1 was in a complex with adenylyl cyclase and that overexpression of CRY1 reduced cAMP production in response to stimuli. In contrast, knocking out *Cry* genes enhanced cAMP production, increased PKA signaling, and activated the NF-κB B-signaling pathway (25). CRY proteins were shown to bind to the Gs alpha subunit and inhibit its function, thereby attenuating intracellular cAMP, which is dependent on activation by G-protein–coupled receptors (28). These results clearly suggest additional circadian-clock–independent functions of CRY in modulating cellular response to different stimuli, which contributes to our understanding of altered immune response under circadian disruption. In the field of immunometabolism, cellular metabolism in lymphocytes, especially in T cells, is appreciated as an important regulator of lymphocyte function specification and fate that eventually shape an immune response (29–31). Circadian rhythm is known to be involved in the regulation of cell metabolism. Further studies to illustrate the importance of cAMP and metabolic changes in lymphocytes in mediating the autoimmune reaction in *Cry* DKO mice are planned.

The other core clock components, most notably BMAL1, CLOCK, REV-ERBα, and RORα, were reported to be important regulators of immune function and inflammation (8). The key components of the molecular clock, the expression and activity changes of which across the circadian day have direct relationships with important components of the immune system, indicating that circadian rhythm indeed impacts immune cell function, host defense, and inflammation.

C1q is the first component of the classical pathway of complement that plays an important role in the clearance of immune complexes

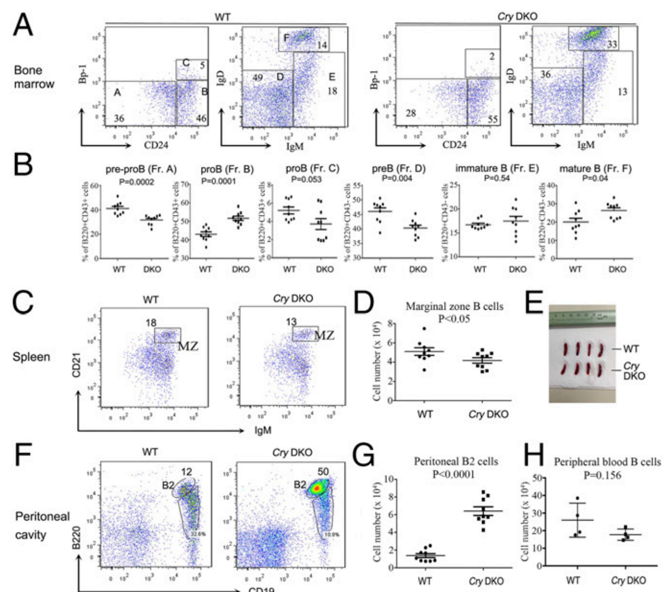

Fig. 4. Flow cytometric and statistical analysis of B cell subsets in *Cry* DKO mice. Cells from nine WT and nine *Cry* DKO mice were used. (*A*) After staining, BM B cell progenitors were classified according to the scheme of Hardy (22) with the following indicated antibodies: fraction A (pre/pro-B cells), fraction B (early pro-B cells), fraction C (late pro-B cells), fraction D (pre-B cells), fraction E (immature B cells) and fraction F (mature B cells). (*B*) Statistical analysis of BM B cell subsets from WT and *Cry* DKO mice. (*C*) Splenic B cell subsets. Splenic B cells were stained with anti-CD19, anti-Thy1.2, anti-IgM, anti-CD93, anti-CD21, and anti-CD23. Percentages of marginal zone B cell subsets (MZ) within the indicated gates are given. (*D*) Marginal zone B cell numbers in spleens of WT and *Cry* DKO mice are provided. Each dot represents data from an individual mouse. (*E*) No difference in spleen size between WT and *Cry* DKO mice. (*F*) Peritoneal cavity cells were stained with anti-CD19, anti-B220, anti-CD5, and anti-CD43. Shown in the gates are the percentages of peritoneal cavity cells in the B2 (CD19+ B220^{high}) B cell subset. (*G*) Peritoneal B2 cell numbers are provided. Each symbol represents data from one mouse. (*H*) Peripheral blood B cells have no change in WT and *Cry* DKO mice.

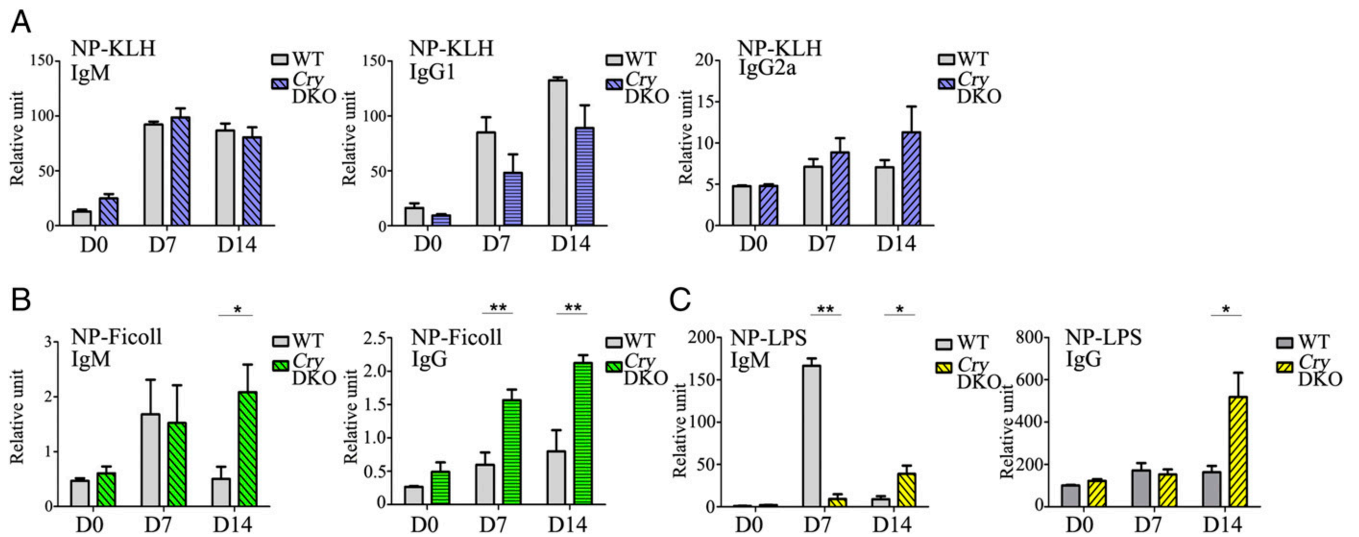

Fig. 5. B cell responses to antigen stimulation in vivo. (*A*) T-dependent antibody responses to NP-KLH in *Cry* DKO mice. Sera were collected from WT and *Cry* DKO mice at days 0, 7, and 14 after immunization. Shown are the titers of NP-specific antibodies in different Ig isotypes. (*B* and *C*) Types I and II T-independent antibody responses. Sera were collected from WT and *Cry* DKO mice at days 7 and 14 after immunization with either NP-Ficoll (*B*) or NP-LPS (*C*). Results in *A*–*C* represent the mean ± SD of three independent experiments, each containing four to five mice of each genotype. *$P < 0.05$; **$P < 0.01$ (two-way ANOVA).

as well as apoptotic bodies, and its dysregulation is considered to be involved in the pathogenesis of SLE (32). Homozygous *C1q* deficiency is the alteration with the strongest disease susceptibility for development of SLE (24). Anti-C1q antibodies are significantly higher in SLE patients and are associated with SLE global activity (33). We found that levels of *C1qa*, *C1qb*, and *C1qc* were significantly down-regulated in *Cry* DKO spleen B cells (Fig. 6), indicating one possible cause of autoimmunity in *Cry* DKO mice.

Protective immunity against a broad range of pathogens requires the ongoing generation of lymphocytes with diverse antigen receptor specificities, which is achieved via random assortment of germline-encoded V(D)J genes in developing lymphocytes. Autoreactive receptors are created during the process with the potential to elicit an autoimmune response. We showed higher V(D)J gene recombination in *Cry* DKO pre-B cells in vitro as well as *Cry* DKO BM cells in vivo. B cell-mediated autoimmunity is frequently linked to the hyperactivation of B cells. In this regard, mice deficient in the major negative regulators of BCR [tyrosine phosphatase SHP-1 (34) or membrane receptor CD22 (35)] exhibit B cell hyper-responsiveness upon stimulation, resulting in systemic autoimmune diseases. By regulating B cell survival and tolerance, BCR signaling both defines and refines the mature, naive compartment. While BCR signals serve as the dominant pathway, additional survival signals, mediated by CD40, BAFF-R,

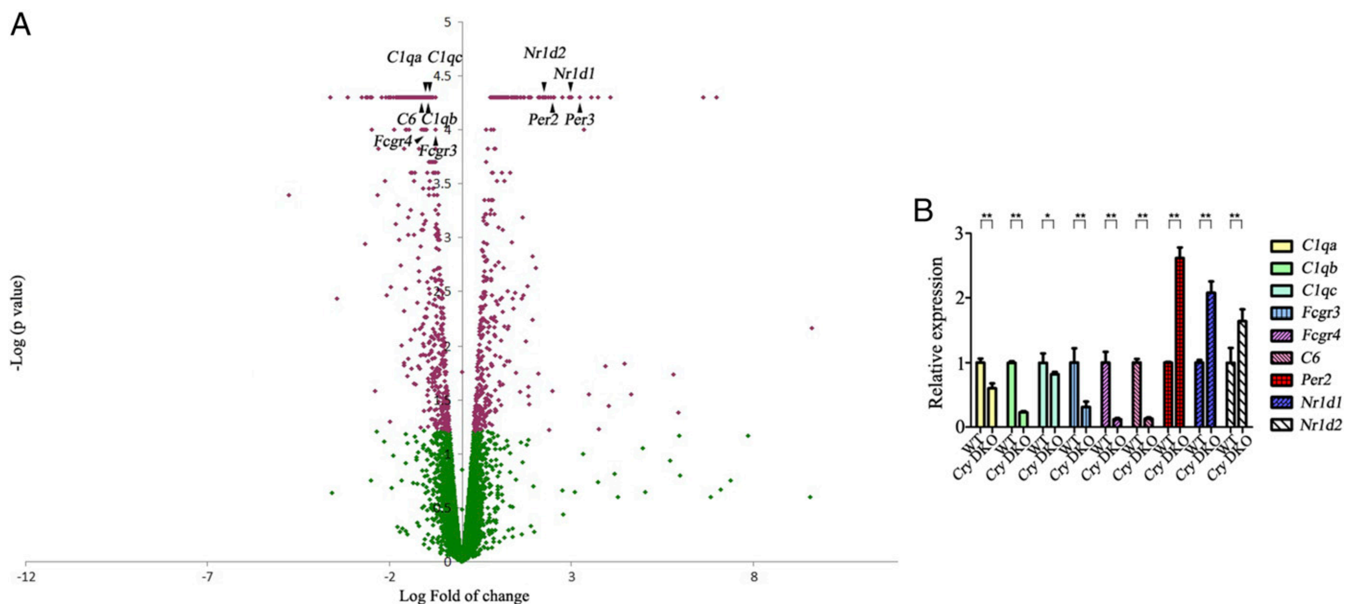

Fig. 6. *Cry* DKO splenic B cells have decreased levels of *C1q*. RNA was extracted from sorted CD19+ *Cry* WT and DKO spleen B cells and used for RNA sequencing. (*A*) Volcano plot illustrates fold-change (*x* axis) and statistically significant distribution (*y* axis) of the gene expression dataset in *Cry* DKO mice versus WT, highlighting the most significantly differentially expressed genes associated with SLE. *C1qa*, *C1qb*, *C1qc*, *C6*, *Fcgr3*, and *Fcgr4* were down-regulated, and circadian clock genes *Per2*, *Per3*, *Nr1d1*, and *Nr1d2* were up-regulated in the *Cry* DKO cells with *Cry* knocked down. (*B*) Expression levels of indicated genes were confirmed by qPCR in CD19+ *Cry* WT and DKO splenic B cells. Data are means ± SD of triplicate experiments. *$P < 0.05$; **$P < 0.01$ (two-tailed, unpaired *t* test).

TACI, and TLR receptors, serve to fine-tune which transitional B cells are able to compete and persist within the naive compartment (36). Here, we showed that the splenic B cells from *Cry* DKO mice have markedly enhanced tyrosine phosphorylation of cellular proteins compared with the WT B cells (Fig. 3 *E* and *F*). However, the molecular mechanisms by which CRY proteins regulate signaling of BCR remain unclear.

Tregs play a fundamental role in inhibiting self-reactivity and maintaining immune tolerance (37, 38). Development of autoimmunity may reflect alterations in both effector T cells (Teffs) and Tregs, reflecting the fact that a proper balance of Teffs and Tregs is critical to achieve and maintain peripheral tolerance (39). In humans, defects in the number, phenotype, and/or function of Tregs have been described in many autoimmune diseases, including type 1 diabetes mellitus and multiple sclerosis (40). Nevertheless, our *Cry* DKO mice had normal levels of Foxp3+ Treg cells compared with WT mice (Fig. S2).

As the molecular clock may regulate many aspects of our immune system, an understanding of how the clock proteins and immune function intersect may reveal therapeutic approaches for some of our most common chronic diseases including immune diseases.

Materials and Methods

Mice. $Cry1^{-/-}Cry2^{-/-}$ mice were a kind gift from Aziz Sancar, University of North Carolina School of Medicine, Chapel Hill, NC (17). All animal care and treatments were in accordance with the Salk Institute guidelines.

RNA Sequencing and Data Analysis. CD19+ spleen B cells were sorted by Aria II (BD Science) with biological duplicates for all treatments. Total RNA was isolated with a RNeasy mini kit (Qiagen). RNA purity and integrity were confirmed using an Agilent Bioanalyzer. Libraries were prepared from 100 ng total RNA (TrueSeq v2; Illumina), and singled-end sequencing was performed on Illumina HiSEq. 2500, using bar-coded multiplexing and a 100-bp read length, yielding a median of 34.1 M reads per sample. Read alignment and junctional finding were accomplished using STAR (41) and differential gene expression with Cuffdiff 2 (42), using the University of California, Santa Cruz mm 9 as the reference sequence. All microarray and RNA sequencing data are available in the GEO database (GSE87467).

Statistical Analyses. The Student's *t* test (unpaired *t* test) or two-way ANOVAs were used to analyze statistical significance, and normality tests were employed to examine the assumption of a normal distribution. GraphPad Prism software was used for statistical calculations. Error was calculated using SD unless otherwise noted; $*P < 0.05$ and $**P < 0.01$.

Extended materials and methods are provided in *SI Materials and Methods*.

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