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 Cry\textsubscript{1} and Cry\textsubscript{2} (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 autoimmune 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 (Per\textsubscript{1}, Per\textsubscript{2}, and Per\textsubscript{3}) and Cryptochrome (Cry\textsubscript{1} and Cry\textsubscript{2}), which in turn repress BMAL1 and CLOCK activity (3). “Reverse orientation c-eb” (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\textsuperscript{+} T helper (Th\textsubscript{17}) 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 Reverb\textsubscript{a}– mice (10). Furthermore, macrophages from murine spleens, lymph nodes, and peritoneum produce different levels of inflammatory cytokines and chemokines at different times of day. Thus, circadian oscillations play an important role in host defense (11).

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\textsuperscript{+} T helper (Th\textsubscript{17}) cells. In this study, we show that mice deficient of the circadian clock genes Cry\textsubscript{1} and Cry\textsubscript{2} 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 functions 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 immunodeficiency, 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-γ2 (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 Cry1 and Cry2 (16). Modern life often involves chronic circadian disruptions, such as night shift work or jet lag which 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. 1A). Cry DKO mice have a substantially (five- to sixfold) higher level of serum IgG antibodies compared with WT CS7BL/6j (B6) mice, while the serum levels of IgM were similar between Cry DKO and WT mice (Fig. 1B). 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, FANA in the sera of Cry DKO mice compared with WT mice (Fig. 1C), 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. 1D). Furthermore, electron microscopic examination showed massive immune complex deposits in the mesangium of the kidneys of Cry DKO mice (Fig. 1E). 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. 1F, 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. 1G 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. 1I).

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 1,000× magnification. Immunofluorescent analyses 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. (E) 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 < 0.01, ***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. 3A). 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. 3B). Vπ-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 λ light chains on the cell surface (Fig. 3C). Notably, the percentage of cells with λ light chain expression was markedly increased in Cry DKO compared with WT cells (Fig. 3C 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. 3E). 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. 3F). Taken together, these results show that loss of CRY proteins affects the strength of multiple BCR-proximal signaling 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. 4A, 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. 4E). Also, the percentage of B cells in peripheral blood was not significantly different between WT and Cry DKO mice (Fig. 4H). 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-lipopolysaccharide (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
DKO mice had C1qc and C1qb in PNAS. DKO mice are labeled. (Fig. 3). DKO cells were RNA-sequenced and found to have heightened inflammatory joint responses, the presence of serum antinuclear antibodies, and the presence of IgG, IgM, and C3 in their glomeruli (Figs. 1 and 2). 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. 6A), which was confirmed by qPCR (Fig. 6B).

**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 C5 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 to potentiate the immune system and to potentiate the immune system and to potentiate the immune system and to potentiate the immune system and to potentiate the immune system and to potentiate the immune system and to potentiate the immune system and to potentiate the immune system. These results clearly 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. 6A), which was confirmed by qPCR (Fig. 6B).

**Flow cytometric and statistical analysis of B cell subsets in CRY DKO mice.** Cells from nine WT and nine CRY DKO mice were used. Similar to CRY1, 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 Gα12 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-dependent 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 immunomeatabolism, 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 in a complex with adenyl 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 Gα12 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-dependent 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 immunomeatabolism, 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.
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,
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 single-ended 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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