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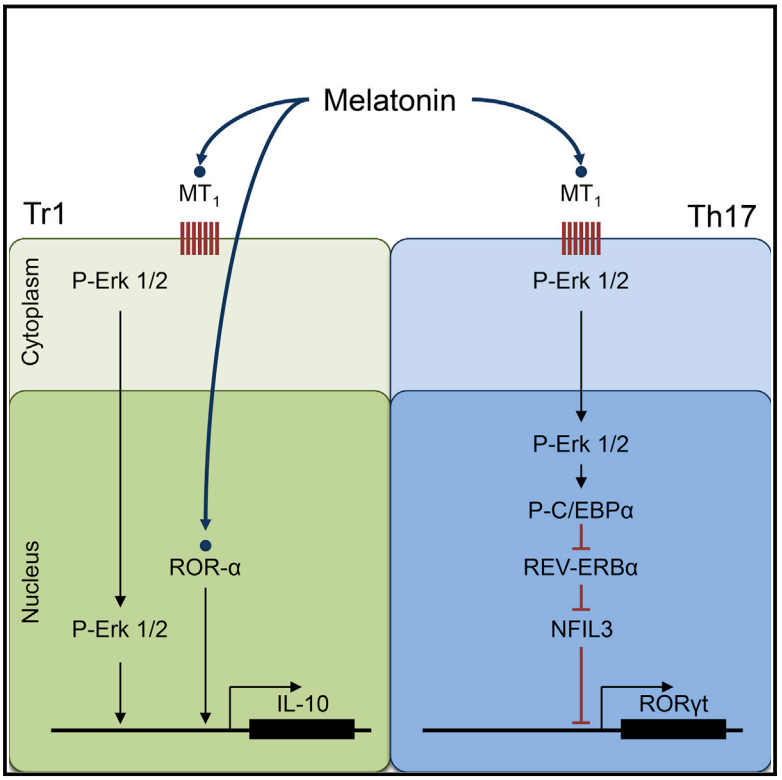
Melatonin Contributes to the Seasonality of Multiple Sclerosis Relapses

Graphical Abstract



Highlights

- Melatonin levels negatively correlate with multiple sclerosis relapses in humans
- Melatonin treatment ameliorates pathology in a mouse model of multiple sclerosis
- Melatonin blocks ROR-γt expression and Th17 differentiation
- Melatonin boosts Tr1 development via Erk1/2 and ROR-α

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In Brief

Melatonin affects the differentiation and function of effector and regulatory T cells in vitro and in vivo, representing an environmental cue that contributes to the seasonality of multiple sclerosis relapses and a potential target for therapeutic intervention in immune-mediated diseases.

Farez et al., 2015, Cell *162*, 1338–1352
September 10, 2015 ©2015 Elsevier Inc.
http://dx.doi.org/10.1016/j.cell.2015.08.025

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Melatonin Contributes to the Seasonality of Multiple Sclerosis Relapses

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http://dx.doi.org/10.1016/j.cell.2015.08.025

SUMMARY

Seasonal changes in disease activity have been observed in multiple sclerosis, an autoimmune disorder that affects the CNS. These epidemiological observations suggest that environmental factors influence the disease course. Here, we report that melatonin levels, whose production is modulated by seasonal variations in night length, negatively correlate with multiple sclerosis activity in humans. Treatment with melatonin ameliorates disease in an experimental model of multiple sclerosis and directly interferes with the differentiation of human and mouse T cells. Melatonin induces the expression of the repressor transcription factor Nfil3, blocking the differentiation of pathogenic Th17 cells and boosts the generation of protective Tr1 cells via Erk1/2 and the transactivation of the IL-10 promoter by ROR-α. These results suggest that melatonin is another example of how environmental-driven cues can impact T cell differentiation and have implications for autoimmune disorders such as multiple sclerosis.

INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated disease of the CNS that is thought to result from the destruction of myelin by autoreactive T cells. $CD4^+$ T cells characterized by the production of IFN-γ (Th1 cells) or IL-17 (Th17 cells) are considered important contributors to MS immunopathogenesis (Miossec et al., 2009; Sospedra and Martin, 2005; Steinman, 2014). $FoxP3^+$ regulatory T cells (Tregs) and IL-10-secreting type 1 regulatory T cells (Tr1) regulate the activity of effector T cells, accordingly, deficits in Tregs and Tr1 cells have been described in MS (Astier et al., 2006; Sakaguchi et al., 2010; Viglietta et al., 2004). Thus, the balance between effector and regulatory T cells controls MS disease activity (Miossec et al., 2009; Sospedra and Martin, 2005; Steinman, 2014).

Genetic polymorphisms have been associated with MS risk and/or pathogenesis (Beecham et al., 2013; Sawcer et al., 2011). However, environmental factors such as infections (Ascherio et al., 2001; Correale and Farez, 2007; Correale et al., 2006), sodium intake (Farez et al., 2014), smoking (Hernán et al., 2005), and vitamin D levels (Ascherio et al., 2014) are also known to affect MS development and course. Lower levels of vitamin D, for example, are associated with higher relapse rates (Runia et al., 2012; Simpson et al., 2010). As a result of the regulation of its synthesis by sun exposure, a significant seasonal fluctuation on vitamin D levels is observed in most locations, with a peak in spring-summer and a nadir in autumn and winter (Rosecrans and Dohnal, 2014). Thus, based on the reported anti-inflammatory effects of vitamin D (Correale et al., 2009) (Ascherio et al., 2010), MS relapse occurrence is predicted to peak during autumn and winter. However, several studies, including a meta-analysis (Jin et al., 2000) and a recent multicentric study (Spelman et al., 2014) found that MS disease activity is higher in spring and summer, suggesting that additional factors play a role in MS relapse seasonality.

Here, we report that melatonin levels, which peak in autumn-winter, show an inverse correlation with clinical disease activity in MS patients. Moreover, melatonin limits the development of experimental autoimmune encephalitis (EAE) and controls Th17 and Tr1 cell differentiation. Thus, seasonal changes in melatonin levels may contribute to the decreased disease activity observed in autumn and winter through a mechanism mediated, at least partially, by the regulation of effector and regulatory T cells.

RESULTS

Melatonin Levels Are Negatively Correlated with MS Clinical Relapses

We first established the seasonality of MS relapses in our cohort of 139 relapsing remitting MS patients (Table 1). Using a Poisson

Table 1. Baseline and Clinical Characteristics of the Study Population

	All Participants (n = 139)
Age (years, mean ± SD)	38.6 ± 10.9
F:M (n)	87:52
Disease duration (years, median, range)	6 (1–20)
EDSS (median, range)	1 (0–4)
Treatment (n)	
None	2
Interferon	64
Glatiramer acetate	34
Natalizumab	2
Fingolimod	26
Other	11
6-SM levels (ng/mg creatinine, mean ± SEM)	
Summer	19.8 ± 1.5
Fall	21.8 ± 1.6
Winter	24.7 ± 0.6
Spring	19.2 ± 1.7
Vitamin D levels (ng/ml)	
Summer	27.8 ± 0.8
Fall	25.2 ± 0.1
Winter	21.7 ± 3.2
Spring	21.7 ± 3.3

regression model, we detected a 32% reduction in the number of relapses occurring during fall and winter (incidence rate-ratio [IRR] 0.682, 95% confidence interval [CI] 0.49–0.95, p = 0.02). Hence, the MS patient cohort used in this study shows the seasonality of MS relapses previously described for other cohorts (Jin et al., 2000; Spelman et al., 2014).

Melatonin production is stimulated by darkness and follows a seasonal pattern with higher levels during fall and winter (Brzezinski, 1997). Melatonin impacts several biological processes, including the circadian clock and the immune response (Brzezinski, 1997). Thus, we investigated the relationship between melatonin and MS disease activity by measuring 6-sulfatoxymelatonin (6-SM) levels in relapsing-remitting MS patients. Since 6-SM is the main melatonin metabolite, its levels in first morning urine are strongly correlated with nighttime melatonin secretion, supporting its use in epidemiological studies (Graham et al., 1998; McMullan et al., 2013). In agreement with previous reports (Morera and Abreu, 2007; Ueno-Towatari et al., 2007), we detected increased melatonin secretion during fall and winter, with lower levels during spring and summer (Figure 1A; Table 1). Moreover, we found a significant negative correlation between 6-SM levels and MS exacerbation rates ($p < 0.01$ Spearman's correlation). This was further confirmed in an age and gender-adjusted Poisson regression model, with a 3% reduction in the number of relapses for each 6-SM unit increase (IRR 0.97, 95% CI 0.95–0.99, p = 0.007). Finally, to test whether the relationship between melatonin levels and exacerbation rate was synchronous, we lagged the occurrence of relapses for 1 (IRR 1.01, 95% CI 0.97–1.05; p = 0.7), 2 (IRR 1.03, 95% CI 0.99–1.07; p = 0.1), and 3 months (IRR 1.03, 95% CI 0.99–1.07; p = 0.7), with no evidence of a lagged effect in relapse occurrence.

We also assessed vitamin D levels and, as previously reported for healthy controls and MS patients in our region (Correale et al., 2009; Fassi et al., 2003), overall levels were low throughout the year with higher levels during summer but no significant correlation with MS relapses (Figure 1B). Finally, we did not detect a correlation between MS relapses and additional environmental factors such as reported upper respiratory tract infections and UV incidence, as determined by national registries and NASA satellites, respectively (Figures 1C and 1D). Thus, higher melatonin levels during fall and winter are associated with a reduction in clinical relapses.

Melatonin Ameliorates Experimental Autoimmune Encephalitis

Based on our epidemiological findings, we studied the effects of melatonin on CNS inflammation using the EAE model of MS. Naive C57BL/6 wild-type mice were immunized with MOG_{35-55} and treated daily with melatonin (5 mg/kg, intraperitoneally) or vehicle. Melatonin administration ameliorated EAE clinical symptoms (Figures 2A and S1A; Table S1). The amelioration of EAE was associated with a decreased number and frequency of Th17 cells in spleen, lymph nodes, and CNS; this decrease was also detected in IL-17$^+$ IFNγ^+ and IL-17$^+$ GM-CSF$^+$ CD4$^+$ T cells that have been associated to the pathogenesis of EAE (Codarri et al., 2011; El-Behi et al., 2011; Lee et al., 2012) (Figures 2C and 2D). We also detected a concomitant increase in IL-10 secreting CD4$^+$ T cells; no significant changes were detected in the number or frequency of other T cell subsets, B cells, $\gamma\delta$ T cells, or innate lymphoid cells (ILCs) (Figures 2B and S1B–S1D).

To further characterize the effects of melatonin on the encephalitogenic T cell response, we analyzed the recall response to MOG_{35-55}. Splenocytes from melatonin-treated mice showed a diminished proliferative response to MOG_{35-55}, reduced IL-17 concomitant with increased IL-10 production, however, no significant effects were detected on IFN-γ production (Figures 2E and 2F). Thus, melatonin arrests the encephalitogenic Th17 cell response.

To investigate if melatonin acts directly on T cells or whether it controls the T cell response indirectly through its effects on antigen presenting cells, we co-incubated sorted CD4$^+$ T cells from melatonin-treated or control mice with treatment-switched dendritic cells (DCs). When compared to controls isolated from vehicle-treated mice, CD4$^+$ T cells from melatonin-treated mice co-incubated with splenic DCs isolated from control mice showed decreased proliferation and IL-17 secretion, concomitant with increased IL-10 production, (Figures 2G and 2H). Conversely, we did not detect significant differences when we used DCs isolated from melatonin or vehicle-treated mice to activate CD4$^+$ T cells from control-treated mice.

In support for a direct effect of melatonin on T cells, melatonin suppressed the in vitro activation of naive 2D2$^+$ transgenic T cells with MOG_{35-55} and DCs (Figures 2I and S1E) or with antibodies

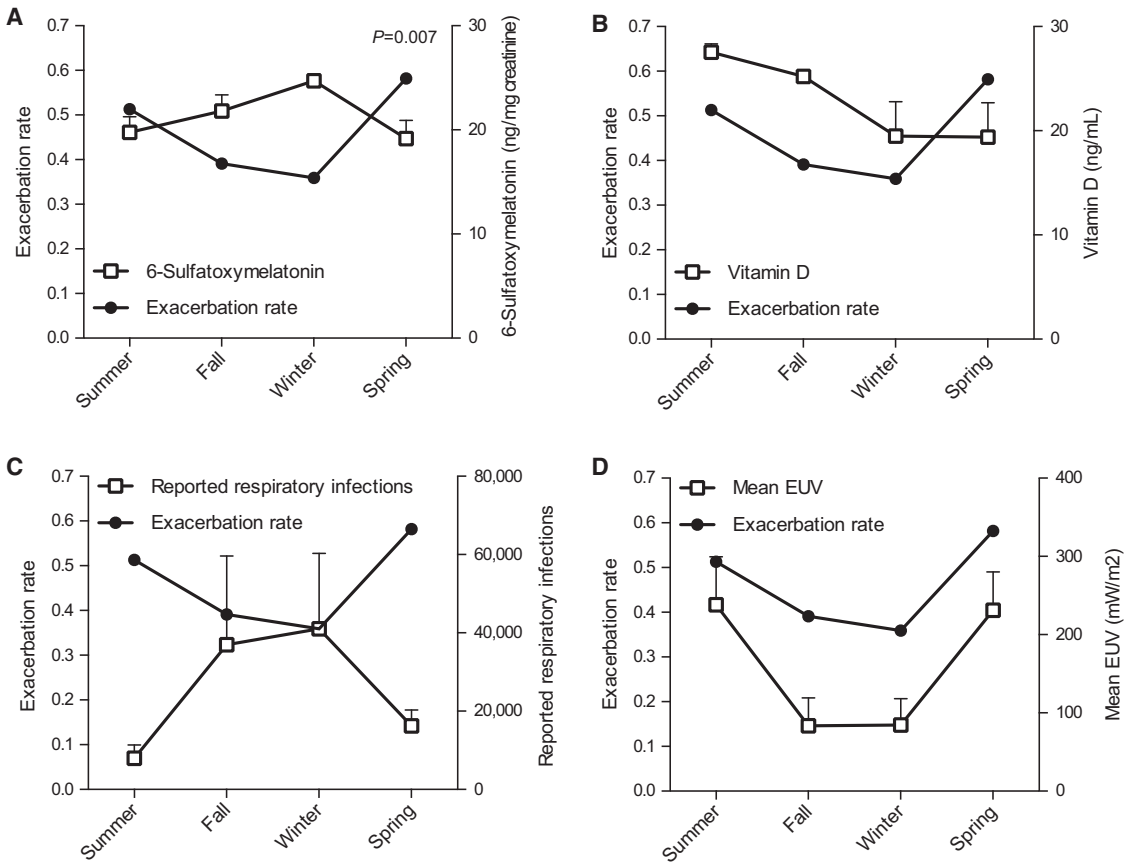

Figure 1. Melatonin Levels Show an Inverse Correlation with MS Clinical Relapses

(A) Exacerbation rate for each season was estimated for the duration of the follow-up and depicted in the primary axis. 6-sulfatoxymelatonin levels measured in first morning urine in each season is depicted as mean ± SEM in secondary axis. p value corresponds to Poisson regression model.

(B–D) Lack of correlation between exacerbation rate and vitamin D (B), reported respiratory infections (C), and UV radiation in Buenos Aires city (D).

See also Table 1.

to CD3 and CD28 in the absence of DCs (Figure 2J). Pretreatment of DCs with melatonin did not affect their ability to activate 2D2$^+$ T cells in the presence of $MOG_{35–55}$ (Figure 2K). Melatonin did not increase apoptosis in CD4$^+$ T cells stimulated with antibodies against CD3 and CD28, as indicated by the analysis of annexin V and propidium iodide staining by flow cytometry or the expression of Bcl-xl levels (Figures S1F and S1G). IL-10 blockade, however, abrogated the suppressive effects of melatonin on T cell proliferation (Figure S1H).

Melatonin Affects Human T Cell Differentiation

We then studied the effects of melatonin on human CD4$^+$ T cells. In addition, we also analyzed the effects of agomelatine, which activates melatonin-dependent signaling (Hickie and Rogers, 2011). Based on the effects of melatonin administration on T cells during EAE, we focused our studies on human Th17 and Tr1 cells. Melatonin and agomelatine reduced the production of IL-17, *RORC*, and *IL17A* expression by human CD4$^+$ T cells activated under Th17 polarizing conditions (Figures 3A–3C and S2), no effect was detected on the differentiation of human Th1 cells (Figures 3D–3F). Concomitantly, melatonin and agomelatine increased *IL10* expression. Indeed, melatonin and agomelatine also increased IL-10 production by human CD4$^+$ T cells activated under Tr1 polarizing conditions (Figures 3G and 3H).

To further investigate the role of melatonin on the immune response in MS, we analyzed the correlation between serum melatonin levels and *IL17* and *IL10* expression in peripheral CD4$^+$ T cells of 26 RRMS patients (Table S2). Using an age- and gender-adjusted linear regression model, we detected a negative correlation between melatonin in serum and *IL17* expression in peripheral CD4$^+$ T cells ($p = 0.012$): higher serum melatonin levels were associated to lower *IL17* expression (Table S3). Conversely, linear regression analysis identified a positive correlation between higher *IL10* expression in peripheral CD4$^+$ T cells and melatonin in serum ($p = 0.003$). We did not detect a significant correlation between melatonin levels and the expression of *RORC*, *NR1D1*, or *NFIL3* in CD4$^+$ T cells (Table S3). Thus, melatonin modulates the differentiation of human Th17 and Tr1 cells in vitro, and endogenous melatonin levels are associated to the expression levels of *IL17* and *IL10* in peripheral CD4$^+$ T cells in RRMS patients.

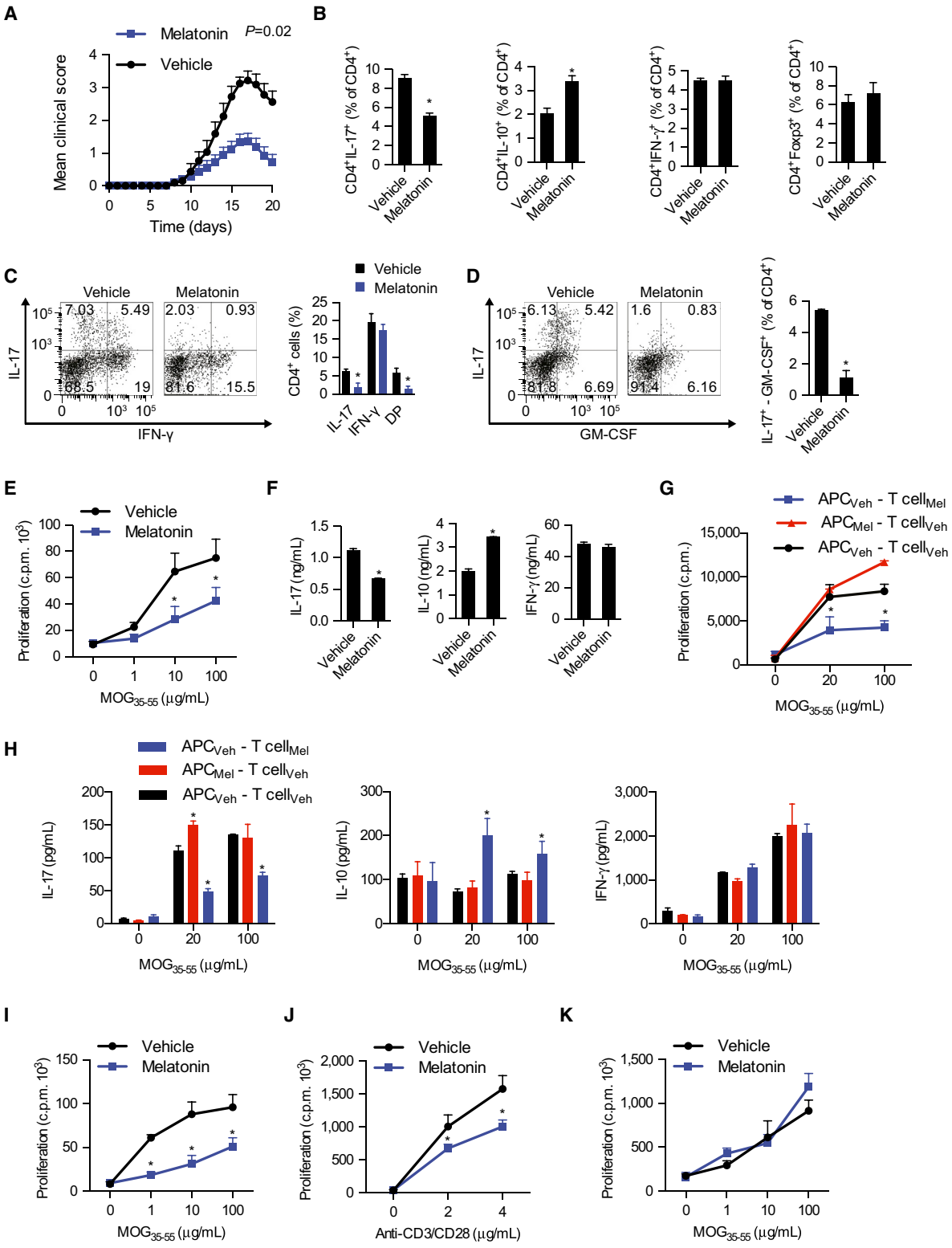

Figure 2. Melatonin Administration Ameliorates EAE

(A) EAE development in C57BL/6 treated with vehicle (0.01% DMSO) or melatonin (5 mg/kg). Data are representative of three independent experiments (means and SEM) (n ≥ 20 mice/group). p value corresponds for the effect of treatment in a repeated-measures mixed effect model.

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Melatonin Interferes with Th17 Generation

Together with Th1 cells, Th17 cells promote the development of EAE and are thought to contribute to MS pathogenesis (Korn et al., 2009). Based on the suppressive effects of melatonin on EAE and IL-17 production by $CD4^+$ T cells, we studied the effects of melatonin on murine Th17 cell differentiation. Melatonin interfered with the differentiation of Th17 cells in vitro as indicated by the expression of rorc, IL-17, and the IL-23 receptor necessary for the differentiation of Th17 cells into fully pathogenic cells; no effects were detected on the differentiation of $FoxP3^+$ iTregs, Th1, or Th2 cells. (Figures 4A, 4B, and S3) (Lee et al., 2012). Melatonin also increased the expression of IL-10, associated to non-pathogenic Th17 cells (Lee et al., 2012; McGeachy et al., 2007) (Figures 4A and 4B).

IFNγ and IL-2 have been shown to limit Th17 cell differentiation (Korn et al., 2009). However, in our studies Th17 cells were differentiated in the presence of IFNγ-blocking antibodies, and IL-2 blocking antibodies failed to abrogate the suppression of Th17 differentiation by melatonin (Figure S4A and S4B). Thus, melatonin suppresses Th17 cell differentiation through a mechanism independent of IFNγ or IL-2.

Physiological concentrations of melatonin result in the activation of signaling pathways controlled by membrane and nuclear receptors (Brzezinski, 1997). The melatonin membrane receptor MTNR1A is expressed by a variety of tissues including cells of the immune system (Jockers et al., 2008; Pozo et al., 1997). In addition, melatonin binds to the nuclear retinoid-related orphan receptor alpha (ROR-α), which is also expressed by immune cells (Pozo et al., 2004) and plays a role in Th17 development (Yang et al., 2008). We detected the expression of both MTNR1A and ROR-α on Th17 cells (Figures S4C and S4D). To study the role of MTNR1A signaling on the effects of melatonin on Th17 cells, we used the MTNR1A-specific agonists agomelatine and ramelteon (Karim et al., 2006) (Figure S4E). Similar to our observations with melatonin, MTNR1A activation by agomelatine or ramelteon suppressed the differentiation of Th17 cells (Figures 4C, 4D, S4F, and S4G). Conversely, melatonin failed to suppress the differentiation of MTNR1A-deficient (MTNR1A KO) Th17 cells (Figures 4E and 4F). Thus, MTNR1A mediates the suppressive effects of melatonin on Th17 cell differentiation.

Melatonin Suppresses Th17 Cell Differentiation via Erk1/2 and C/EBPα Activation

REV-ERBα (encoded by nr1d1) is a component of the circadian clock that promotes Th17 differentiation by limiting the expression of NFIL3, a direct inhibitor of rorc transcription (Yu et al., 2013). Melatonin regulates the activity of both circadian and seasonal clocks (Pévet, 2003). Indeed, melatonin levels show a circadian inverse correlation with nr1d1 expression, suggesting that melatonin affects REV-ERBα expression (Kojetin and Burris, 2014). Thus, we investigated whether melatonin acts on REV-ERBα to suppress Th17 cell differentiation.

Using reverse protein arrays (Farez et al., 2009) we analyzed signaling pathways triggered by melatonin in T cells and detected an MTNR1A-dependent increase in the activation of Erk1/2 (Figures 4G, 4H, S4H, and S4I). Of note, Erk1/2 inhibition has been previously shown to enhance Th17 cell differentiation (Tan and Lam, 2010) and Erk1/2 phosphorylation has been linked to the reduced expression of REV-ERB proteins (Castellano et al., 2014; Kojetin and Burris, 2014), but the mechanism involved and its relevance for T cells has not been characterized yet. Through a bioinformatic analysis of the nr1d1 promoter, we identified a binding site for the CAAT/enhancer-binding protein α (C/EBPα), a leucine zipper transcription factor involved in the regulation of cellular differentiation (Lekstrom-Himes and Xanthopoulos, 1998). C/EBPα is a downstream target of Erk1/2 activated by phosphorylation (Johnson, 2005). Thus, we analyzed whether Erk1/2 regulates the transcriptional activity of the nr1d1 promoter in a C/EBPα-dependent manner.

Th17 cell differentiation in the presence of melatonin led to C/EBPα phosphorylation and the recruitment of C/EBPα to the nr1d1 promoter (Figures 4I and 4J). C/EBPα phosphorylation and recruitment to the nr1d1 promoter were suppressed in MTNR1A KO T cells and in the presence of the Erk1/2 inhibitor UO216 (Figures 4I and 4J). Hence, melatonin triggers the recruitment of C/EBPα to the nr1d1 promoter in an MTNR1A- and Erk1/2-dependent manner.

To analyze the effects of C/EBPα on the transcriptional activity of the nr1d1 promoter, we used a reporter construct in which the nr1d1 promoter controls luciferase expression. Treatment of nr1d1 reporter-transfected HEK293 cells with melatonin or agomelatine resulted in decreased luciferase activity and similar

(B) Flow cytometry analysis of $IL\text{-}17^+$, $IL10^+$, IFN-γ^+, and $FoxP3^+$ $CD4^+$ cells from the spleen of vehicle- or melatonin-treated mice at day 7 after disease induction. At least four mice were analyzed per group and data are presented as mean ± SEM. *$p < 0.05$ of unpaired t test.

(C and D) Flow cytometry analysis of $IL\text{-}17^+$, IFN-γ^+, $IL\text{-}17^+$-IFN-γ^+ (DP), and $IL\text{-}17^+$-$GM\text{-}CSF^+$ $CD4^+$ T cells from the CNS of control- or melatonin-treated mice at the clinical peak of EAE. *$p < 0.05$ of unpaired t test.

(E) Proliferative responses of $CD4^+$ T cells to $MOG_{35–55}$ of vehicle- or melatonin-treated mice. At least three mice were analyzed per group and data are presented as mean ± SEM. *$p < 0.05$ of one-way ANOVA.

(F) Cytokine secretion by proliferating $CD4^+$ T cells from vehicle and melatonin-treated. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of unpaired t test.

(G and H) Proliferative responses (G) and cytokine profile (H) of $CD4^+$ T cells in co-culture with dendritic cells derived from melatonin-treated or untreated mice. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(I) Proliferative responses of melatonin-treated 2D2 $CD4^+$ T cells to $MOG_{35–55}$ in the presence of dendritic cells. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(J) Proliferative responses of melatonin-treated 2D2 $CD4^+$ T cells to $MOG_{35–55}$ stimulated only with anti-CD3 and anti-CD28. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(K) Proliferative responses of treated 2D2 $CD4^+$ T cells to $MOG_{35–55}$ stimulated melatonin-treated DCs. Data are representative of three independent experiments (means and SEM).

See also Figure S1 and Table S2.

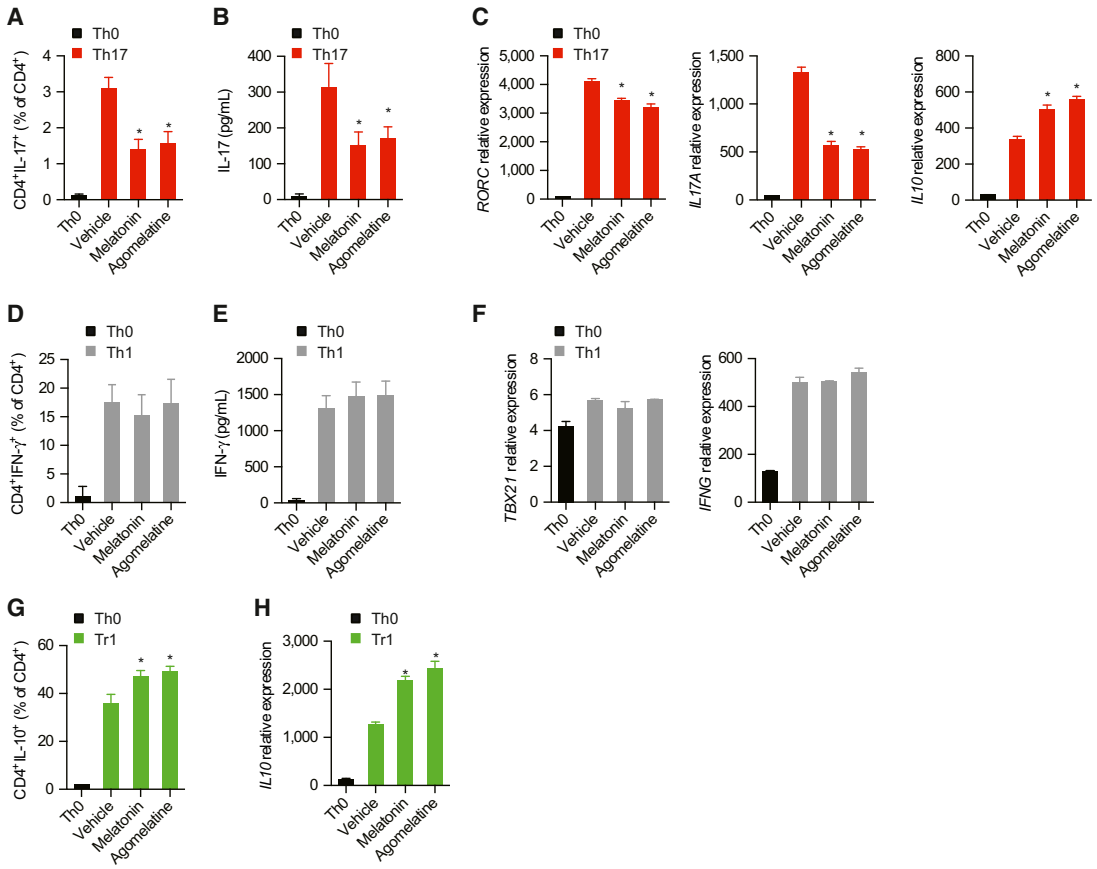


Figure 3. Melatonin Interferes with Human Th17 Cell Differentiation and Boosts Tr1 Generation

(A) Flow cytometry analysis of IL-17 expression in human Th17-differentiated $CD4^+$ T cells (IL-1β, IL-6, and TGF-β1) in the presence or absence of melatonin (500 ng/ml) and agomelatine (500 ng/ml). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(B) Cytokine quantification by ELISA of IL-17 in human Th17-differentiated $CD4^+$ T cells in the presence or absence of melatonin (500 ng/ml) and agomelatine (500 ng/ml). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(C) RT-PCR analysis of Th17 cells cultured as in (A). Data are representative of three independent experiments (means and SEM) *$p < 0.05$ of one-way ANOVA.

(D) Flow cytometry analysis of IFN-γ expression in human Th1-differentiated CD4+ T cells (IL-12) in the presence or absence of melatonin (500 ng/ml) and agomelatine (500 ng/ml). Data are representative of three independent experiments (means and SEM).

(E) Cytokine quantification by ELISA of IFN-γ in human Th1-differentiated $CD4^+$ T cells in the presence or absence of melatonin (500 ng/ml) and agomelatin (500 ng/ml). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(F) RT-PCR analysis of Th1 cells cultured as in (D). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

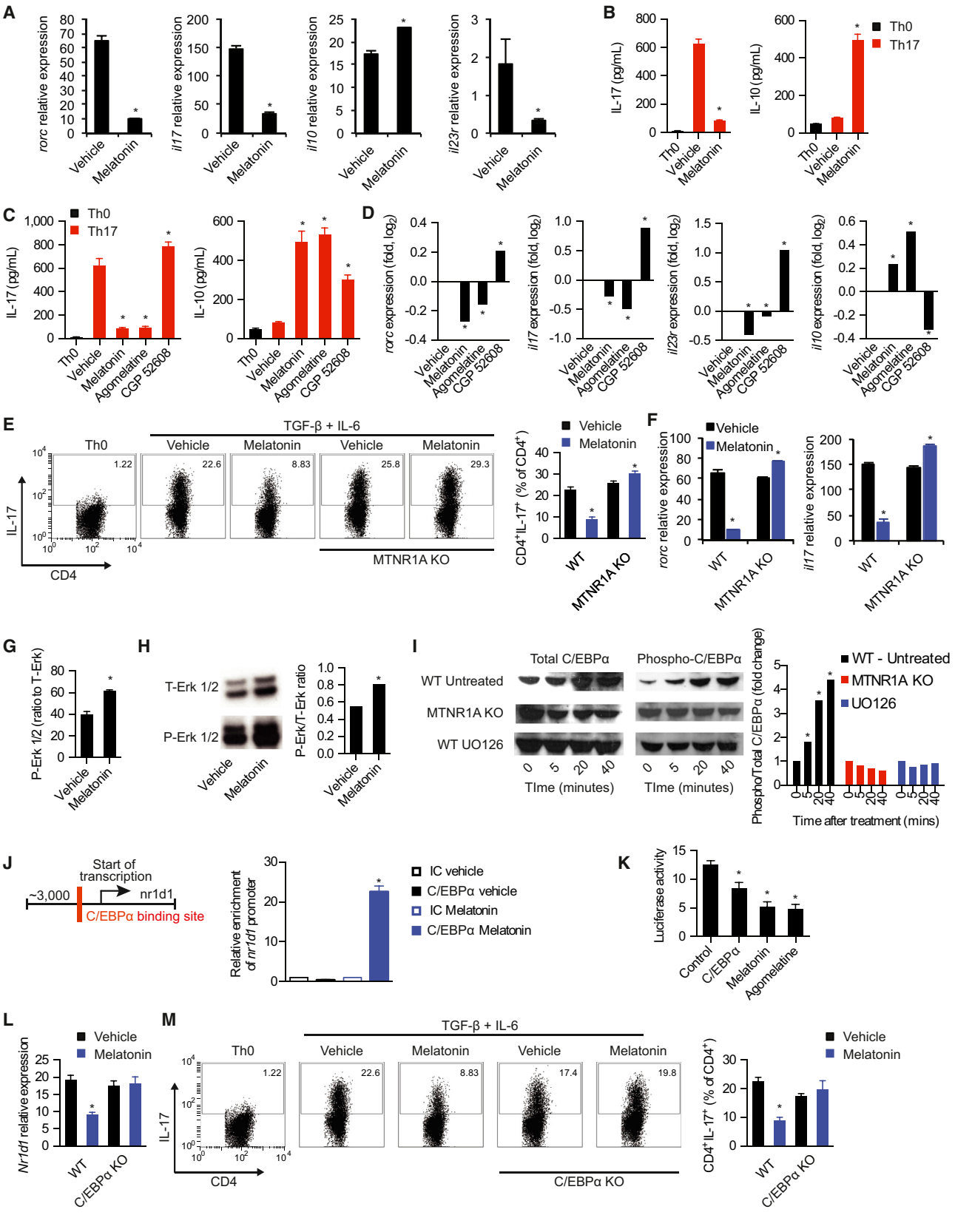
(G) Flow cytometry analysis of IL-10 expression in human Tr1-differentiated $CD4^+$ T cell in the presence or absence of melatonin (500 ng/ml)and agomelatin (500 ng/ml). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(H) RT-PCR analysis of Tr1 cells cultured as in (F). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA. See also Figure S2.

effects were achieved by C/EBPα overexpression (Figure 4K). Finally, to investigate the role of C/EBPα on the suppression of Th17 cell differentiation by melatonin we used C/EBPα-deficient T cells (Yang et al., 2005). C/EBPα-deficiency abrogated the decrease in nr1d1 expression and the suppression of Th17 differentiation induced by melatonin (Figures 4L and 4M). Thus, melatonin suppresses the differentiation of Th17 cells through a mechanism mediated by MTNR1A, Erk1/2, and C/EBPα.

Melatonin Inhibits ROR-γt and ROR-α Expression in Th17 Cells by Inducing Nfil3

NFIL3 limits Th17 cell differentiation by suppressing the expression of ROR-γt (Yu et al., 2013). REV-ERBα inhibits nfil3 expression (Yu et al., 2013). Thus, we hypothesized that the decrease in nr1d1 expression induced by melatonin results in the NFIL3-dependent inhibition of rorc expression (Figure 5A). We detected nr1d1 expression in Th17 cells, but not in Th0 or Tr1 cells (Figure 5B). Melatonin suppressed nr1d1 expression during Th17 cell differentiation, resulting in a concomitant increase in the expression of the ROR-γt repressor NFIL3 (Figures 5C and 5D). In agreement with our results on Th17 cell differentiation, the regulation of REV-ERBα and NFIL3 expression by melatonin was mediated by its membrane receptor MTNR1A and Erk1/2 (Figures 5C–5G). The relevance of the regulation of REV-ERBα expression for the modulation of Th17 cell differentiation by melatonin was confirmed in nr1d1 overexpression experiments



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and by the use of REV-ERBα-deficient T cells. Nr1d1 overexpression and REV-ERBα deficiency abrogated the effects of melatonin on Th17 cell differentiation (Figures 5H–5K). Hence, MTNR1A-dependent signaling triggered by melatonin suppresses Th17 cell differentiation through the regulation of REV-ERBα expression.

ROR-α promotes Th17 cell differentiation (Yang et al., 2008). Accordingly, ROR-α activation by the specific agonist CGP 52608 boosted Th17 cell differentiation (Figures 4C and 4D). ROR-α is directly activated by melatonin (Brzezinski, 1997). Indeed, melatonin boosted the differentiation of MTNR1A-deficient Th17 cells (Figure 4E), suggesting that melatonin-triggered MTNR1A signaling interferes with the promotion of Th17 cell differentiation by ROR-α. Based on the inhibitory effects of NFIL3 on ROR-γt expression and Th17 cell differentiation (Yu et al., 2013), we studied whether NFIL3 also inhibits ROR-α expression.

A bioinformatics analysis identified NFIL3 binding sites in the rora and rorc promoters. Accordingly, we detected the recruitment of NFIL3 to the rora and rorc promoters in $CD4^+$ T cells activated under Th17 polarizing conditions in the presence of melatonin, concomitant with a reduced expression of both ROR-α and ROR-γt (Figures 5L and 5M). We then investigated the relevance of the regulation of NFIL3 expression for the modulation of Th17 cell differentiation. Overexpression of NFIL3 (Figures 5N and 5O) and NFIL3-deficiency (Figures 5P and 5Q) abrogated the suppressive effects of melatonin on Th17 cell differentiation. Thus, the regulation of NFIL3 expression by melatonin mediates its inhibitory effects on the differentiation of Th17 cells in vitro. To evaluate the role of MTNR1A and NFIL3 on the suppression of Th17 cell differentiation by melatonin in vivo, we used RAG-1-deficient mice reconstituted with wild-type, MTNR1A-, REV-ERBα-, or NFIL3-deficient $CD4^+$ T cells and immunized with $MOG_{35–55}$ in CFA. In agreement with our in vitro observations, the suppression of Th17 cell differentiation by melatonin in vivo was abrogated by MTNR1A-, REV-ERBα-, and NFIL3-deficiency (Figures 5R and S5). Indeed, we detected increased Th17 cell differentiation in response to treatment of mice reconstituted with MTNR1A-, REV-ERBα-, or NFIL3-deficient T cells, most likely reflecting the unopposed agonistic activity of melatonin on ROR-α and its promoting effects on the differentiation of Th17 cells. Taken together, these data suggest that melatonin interferes with Th17 cell differentiation via the inhibition of ROR-γt and ROR-α expression through an NFIL3-dependent mechanism.

Melatonin Boosts Tr1 Cell Differentiation via Erk1/2 and ROR-α

$CD4^+$ IL-10-producing Tr1 cells play an important role in the regulation of the immune response (Pot et al., 2011; Roncarolo et al., 2006). The amelioration of EAE by melatonin administration was associated with an increase in IL-10-producing T cells (Figure 2). Thus, we investigated the effects of melatonin on the activation of naive $CD4^+$ T cells under Tr1 polarizing conditions. We found that melatonin boosted the expression of IL-10 and the Tr1-associated molecules il21, ahr, and cmaf (Apetoh et al., 2010) (Figure 6A). In addition, melatonin boosted the suppressive activity of Tr1 cells in vitro (Figure 6B).

We then investigated the mechanisms underlying the effects of melatonin on Tr1 regulatory cells. We detected the expression of both MTNR1A and ROR-α by Tr1 cells (Figures S4C and S4D). Indeed, both agomelatine and CGP 52608, specific agonist for MTNR1A and ROR-α, respectively, boosted Tr1 cell differentiation (Figures 6C and 6D). In agreement with these results, MTNR1A deficiency or inhibition of MTNR1A-activated Erk1/2 by UO126 interfered with the boost in Tr1 differentiation by melatonin (Figures 6E and 6F). Of note, Erk1/2 activation is reported to promote cmaf-dependent IL-10 production by $CD4^+$ T cells (Saraiva et al., 2009). In addition, ROR-α deficiency suppressed the differentiation of Tr1 cells induced by IL-27 and its boost by melatonin (Figure 6G).

Figure 4. Melatonin Interferes with Th17 Cell Differentiation via the Erk1/2-C/EBPα Pathway

(A) $CD4^+$ naive T cells were differentiated into Th17 cells by the addition of TFG-β, IL-6 (0 hr), and IL-23 (48 hr) in the presence or absence of melatonin (2 ng/ml) and analyzed by RT-PCR after 72 hr. Displayed image is representative of five experiments. *$p < 0.05$ of unpaired t test.

(B) Cytokine secretion analysis of IL-17 and IL-10 after 72 hr of culture as in (A). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of unpaired t test.

(C) Cytokine secretion in Th17-differentiated $CD4^+$ T cells in the presence or absence of melatonin (2 ng/ml), agomelatine (20 ng/ml, MTNR1A ligand), and CGP 52608 (20 ng/ml, ROR-α ligand). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(D) RT-PCR analysis of Th17 cells cultured as in (C). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(E) Flow cytometry analysis of IL-17 expression as in (A), in wild-type mice and MTNR1A-deficient mice. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of unpaired t test.

(F) RT-PCR analysis of wild-type and MTNR1A-deficient mice cultured as in (E). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of unpaired t test.

(G) Signal transduction profiling using reverse protein arrays. Data are representative of two independent experiments (means and SEM). *$p < 0.05$ of unpaired t test.

(H) Immunoblot analysis of T-Erk1/2 and P-Erk1/2. Data are representative of two independent experiments (means and SEM).

(I) Immunoblot analysis of T-C/EBPα and P-C/EBPα Data are representative of two independent experiments (means and SEM).

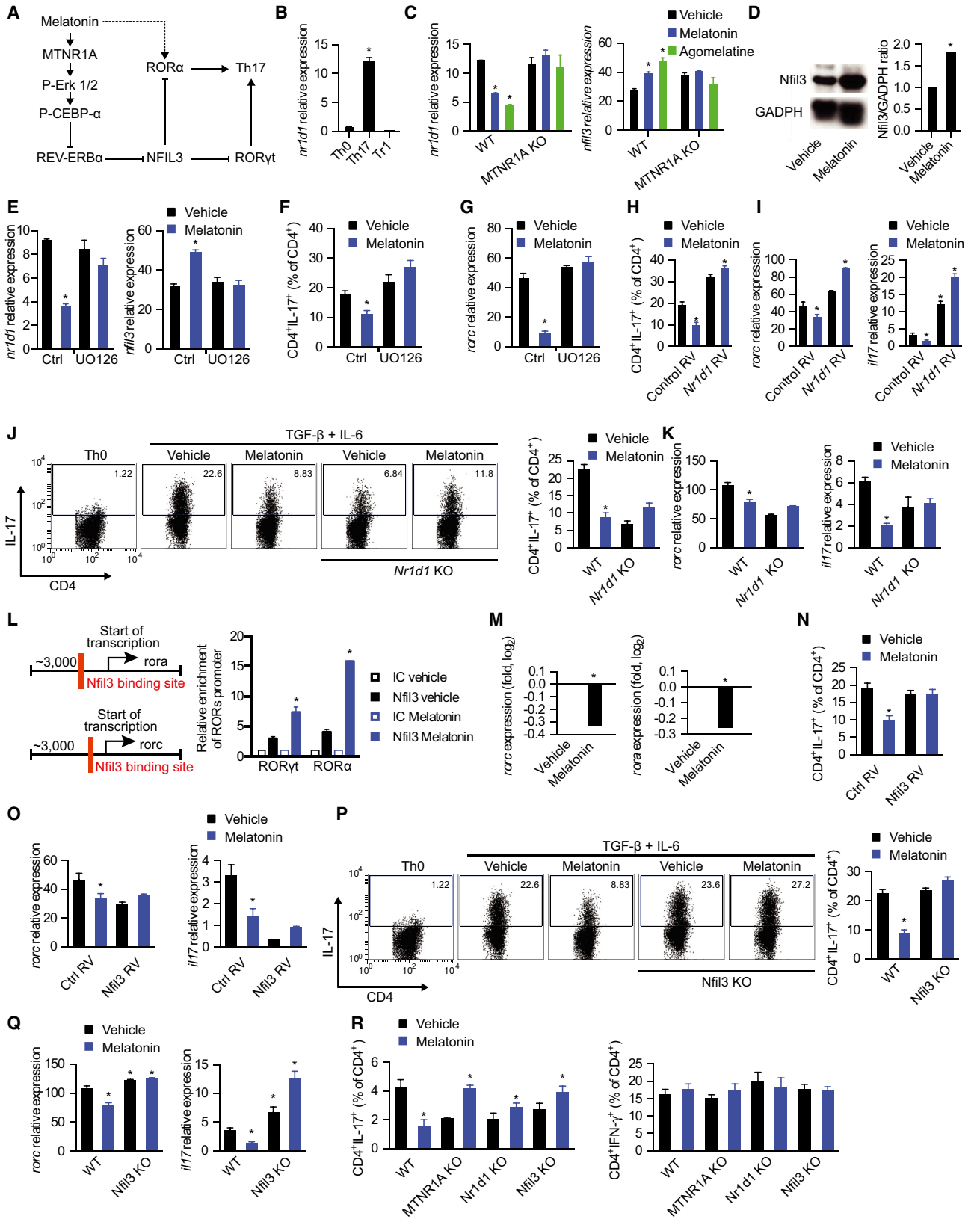
(J) Putative binding sites of C/EBPα in nr1d1 (left); chromatin immunoprecipitation with anti-C/EBPα (right). Data are representative of three independent experiments (means and SEM) *$p < 0.05$ of one-way ANOVA.

(K) Luciferase activity of HEK293 cells transfected with a luciferase reporter construct for the nr1d1 promoter. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of unpaired t test.

(L) Flow cytometry analysis of IL-17 expression as in (A) in wild-type mice and C/EBPα-deficient mice. Data are representative of two independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(M) Flow cytometry analysis of IL-17 expression as in (A), in wild-type mice and C/EBPα-deficient mice. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of unpaired t test.

See also Figures S3 and S4.



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ROR-α exerts its biological effects by binding to ROR response elements (RORES) in target genes (Jetten, 2009). A bioinformatic analysis identified ROR-α binding sites in the il10 promoter (Figure 6H), suggesting that melatonin may increase the recruitment of ROR-α to the il10 promoter and consequently, il10 transcription. In agreement with this hypothesis, we detected increased binding of ROR-α to the il10 promoter following T cell activation under Tr1 polarizing conditions in the presence of melatonin (Figure 6H). Moreover, ROR-α transactivated the il10 promoter in reporter assays and synergized with the aryl hydrocarbon receptor (AhR) and c-Maf to boost their ability to promote il10 expression (Apetoh et al., 2010; Gandhi et al., 2010) (Figure 6I). Taken together, these data suggest that melatonin boosts Tr1 cell differentiation through its effects on MTNR1A and ROR-α (Figure 6J).

DISCUSSION

Strong epidemiological evidence supports the role of vitamin D in reducing MS relapses (Ascherio et al., 2012). Strikingly, vitamin D levels are higher during spring and summer, when relapse occurrence in MS patients peaks. Thus, the observation of a lower occurrence of relapses in seasons characterized by lower vitamin D levels represents a ''seasonal paradox'': relapses should be less frequent in spring and summer when vitamin D levels are higher, yet the opposite is found in most studies (Jin et al., 2000; Spelman et al., 2014), with a few exceptions (Løken-Amsrud et al., 2012). Our data may solve this paradox by identifying melatonin, whose levels are regulated by seasonal fluctuations in day length, as an additional regulator of the immune response in MS. Note that night shift work, which is associated with lower overall melatonin levels (Schernhammer et al., 2004), increases the risk of developing MS (Hedström et al., 2011). These findings suggest that melatonin may also be an MS risk factor; the relationship between melatonin levels and the risk of developing MS is the focus of ongoing investigations. Finally, the interplay between melatonin and other seasonal environmental factors known to impact MS such as vitamin D in different geographic locations remains to be further elucidated.

The rise in the past 50 years in the incidence of autoimmune disorders has reached an epidemic proportion and cannot be accounted by genetic risk only. Thus, increasing attention is being paid to environmental factors and their impact in the immune response and T cell differentiation in particular. For example: several compounds present in household products can activate the aryl hydrocarbon receptor and impact both Th17 and regulatory cell differentiation (Quintana et al., 2008); sodium in westernized diet and processed foods can also enhance Th17 cell differentiation (Wu et al., 2013); the composition of commensal microbiota impacts T cell differentiation and response (Lathrop et al., 2011); and the lack of sun exposure and dietary habits can diminish vitamin D levels and affect regulatory cell function (Correale et al., 2009). Each of these environmental factors trigger different signaling pathways and the characterization of the complex interaction between them can shed light on the impact of the environment on the immune system.

Figure 5. Melatonin Interferes with Th17 Cell Differentiation by Limiting NFIL3 Expression

(A) Schematic diagram of the proposed mechanisms mediating the effects of melatonin on Th17 cell differentiation.

(B) RT-PCR analysis of nr1d1 expression in $CD4^+$ T cells activated under Th0, Th17, and Tr1 polarizing conditions for 3 days. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of unpaired t test.

(C) RT-PCR analysis of nr1d1 (left) and nfil3 (right) expression in $CD4^+$ T cells activated under Th17 polarizing conditions for 3 days treated with vehicle, melatonin (2 ng/ml), or agomelatine (20 ng/ml). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of unpaired t test. NFIL3 expression was further confirmed by western blot.

(D) Immunoblot analysis of Nfil3. Data are representative of two independent experiments (means and SEM).

(E) RT-PCR analysis of nfil3 expression in $CD4^+$ T cells activated under Th17 polarizing conditions for 3 days in the presence of melatonin (2 ng/ml) and/or UO126. Data are representative of five independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(F and G) Flow cytometry analysis of IL-17 expression (F) and rorc expression (G) in $CD4^+$ T cells activated under Th17 polarizing conditions in the presence of melatonin (2 ng/ml) and/or UO126. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(H and I) Flow cytometry analysis of IL-17 expression (H) and rorc and il17 expression (I) in $CD4^+$ T cells activated under Th17 polarizing conditions in the presence of melatonin (2 ng/ml), following infecting with a control or an nr1d1-encoding retrovirus. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(J and K) Flow cytometry analysis of IL-17 expression (J) and rorc and il17 expression (K) in wild-type and REV-ERBα-deficient $CD4^+$ T cells activated under Th17 polarizing conditions in the presence of melatonin (2 ng/ml). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(L) Putative binding sites of Nfil3 in rorc and rora (left). ChIP analysis of the interaction of NFIL3 with its putative binding sites in $CD4^+$ T cells activated under Th17 polarizing conditions (right). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

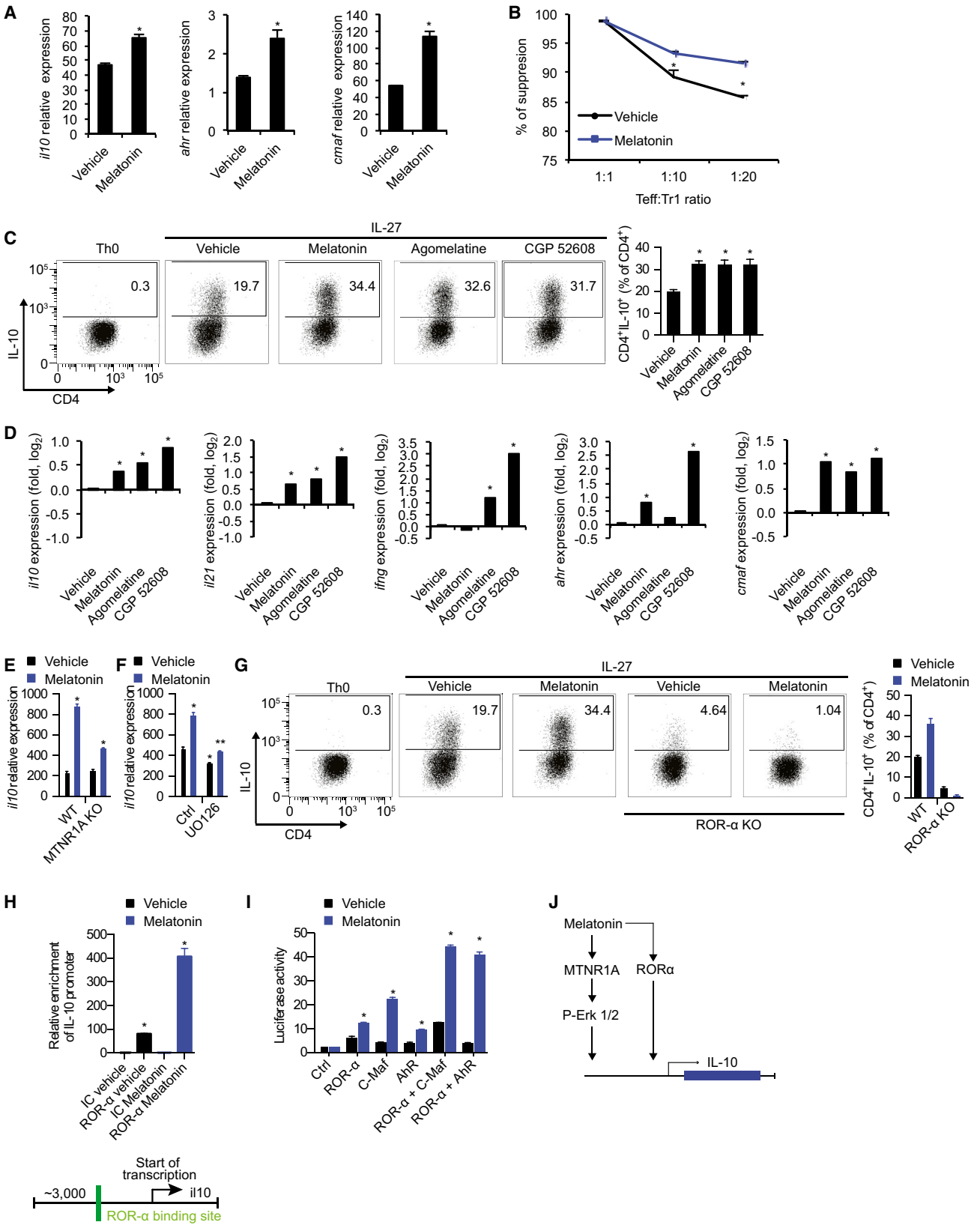
(M) RT-PCR analysis of rorc and rora expression in $CD4^+$ T cells activated under Th17 polarizing conditions in the presence of melatonin (2 ng/ml). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of unpaired t test.

(N and O) Flow cytometry analysis of IL-17 expression (N) and rorc and il17 expression (O) in $CD4^+$ T cells activated under Th17 polarizing conditions in the presence of melatonin (2 ng/ml) and transduced with a control or nfil3-encoding retrovirus. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(P and Q) Flow cytometry analysis of IL-17 expression (P) and rorc and il17 expression (Q) in wild-type mice and NFIL3-deficient in $CD4^+$ T cells activated under Th17 polarizing conditions in the presence of melatonin (2 ng/ml). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(R) Flow cytometry analysis of IL-17 and IFN-γ expression in $CD4^+$ T cells from RAG-1-deficient mice reconstituted with wild-type, MTNR1A- REV-ERBα, or NFIL3-deficient $CD4^+$ T cells, immunized with $MOG_{35–55}$ in CFA and treated with vehicle or melatonin (5 mg/kg). *$p < 0.05$ of unpaired t test.

See also Figure S5.



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Pro-inflammatory Th17 cells are thought to contribute to the pathogenesis of EAE and MS (Miossec et al., 2009). Th17 cell differentiation is regulated by ROR-α and ROR-γt and therapies targeting Th17 cells are currently being tested in MS and other autoimmune diseases with preliminary encouraging results (Baeten and Kuchroo, 2013). Melatonin, despite having the potential to activate ROR-α, suppresses the generation of Th17 cells via its membrane receptor in a NFIL3-dependent fashion. Interestingly, it has been recently shown that the circadian clock suppresses Th17 development during nighttime through a similar NFIL3-dependent mechanism (Yu et al., 2013). Our work suggests that, in addition to Th17 cells, Tr1 cells are also regulated by melatonin during nighttime in an Erk1/2- and ROR-α-dependent manner. Based on the high evolutionary conservation of melatonin production by the pineal gland and its regulation by daylight (Macchi and Bruce, 2004), it is likely that the circadian and seasonal effects of melatonin on the immune response play an important role that resulted in its positive selection during evolution.

Tr1 cells are characterized by the production of IL-10 (Pot et al., 2011; Roncarolo et al., 2006). AhR, c-Maf, and Erk1/2 have been shown to regulate Tr1 cell development and IL-10 expression (Apetoh et al., 2010; Gandhi et al., 2010). Our work shows that melatonin promotes Tr1 cell differentiation by activating Erk1/2 signaling, which has been previously described to control IL-10 expression in T cells and DCs (Saraiva and O'Garra, 2010). We also identified ROR-α as a mediator of the effects of melatonin in Tr1 cells. Thus, these data suggest that melatonin utilizes multiple pathways to boost Tr1 cell differentiation.

The interplay between pro-inflammatory and regulatory cells controls the development of autoimmune diseases such as MS. Here, we report that melatonin, whose levels show seasonal variability, control the balance between pathogenic and regulatory T cells. However, in MS patients, melatonin is likely to act on several cell types to affect disease activity. Indeed, NFIL3 has been shown to play a role in human inflammatory bowel disease and autoimmune colitis through its activity on innate immune cells (Kobayashi et al., 2014). Thus, future studies should investigate the effects of melatonin on innate immune cells in MS patients and also its role in inflammatory bowel disease and other immune-mediated disorders. Finally, although our data identify melatonin-dependent signaling as a potential target for therapeutic immunomodulation, the pathways involved are complex and likely cross-regulated. Thus, extreme caution should be exercised to evaluate the translational potential of these findings.

EXPERIMENTAL PROCEDURES

Patients

Consecutive patients with relapsing-remitting MS according to McDonald criteria (Polman et al., 2011) were recruited from the MS clinic at the Raúl Carrea Institute for Neurological Research (FLENI) between September of 2011 and November of 2012. Study protocol was approved by the Institutional Ethics Committee, and all subjects signed an informed consent form. See Supplemental Experimental Procedures for detailed information.

Animals and EAE

EAE was induced as follows: mice were immunized with 100 μg $MOG_{35–55}$ and 500 μg mycobacterium tuberculosis extract H37Ra (Difco). Mice were also injected intraperitoneally with 200 ng pertussis toxin on days 0 and 2. Melatonin (5 mg/kg) or vehicle (0.01% DMSO) was administered daily at 7:00 p.m.

Flow Cytometry Staining and Acquisition

For intracellular cytokine staining, cells were stimulated for 4 hr at 37°C with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma), ionomycin (1 μg/ml; Sigma), and monensin (GolgiStop; 1 μg/ml; BD Biosciences). After being stained for surface markers, cells were fixed and made permeable according to the manufacturer's instructions (BD Biosciences). All antibodies against cytokines were from Biolegend. All experiments were started at the same time (8:00–9:00 a.m.). Data were collected with a LSR II or FACSAria (BD Biosciences), then were analyzed with FlowJo software (Treestar).

Measurement of Cytokines

Secreted cytokines were measured in tissue culture supernatants after 72–96 hr by ELISA as previously described (Farez et al., 2009).

qRT-PCR

RNA was extracted with RNAeasy columns (QIAGEN), then cDNA was prepared according to the manufacturer's instructions (Applied Biosystems)

Figure 6. Melatonin Boosts Tr1 Cell Differentiation

(A) RT-PCR analysis of il10, ahr, and maf expression in Tr1-differentiated $CD4^+$ T cells in the presence or absence of melatonin (2 ng/ml). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(B) In vitro suppression assay, treated or untreated differentiated Tr1 cells as in a, were co-cultured after 72 hr with $CD4^+$ T cells previously labeled with CSFE, and proliferation cycles (CSFE dilution) were measured after 48 hr by flow cytometry. Data are representative of two independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(C) Flow cytometry analysis of IL-10 expression in Tr1-differentiated $CD4^+$ T cells in the presence or absence of melatonin (2 ng/ml), agomelatine (20 ng/ml, MTNR1A ligand), and CGP 52608 (20 ng/ml, ROR-α ligand). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(D) RT-PCR analysis of Tr1 cells cultured as in (C). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(E) RT-PCR analysis of il10 expression as in (C), in wild-type mice and MTNR1A-deficient mice. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of one-way ANOVA.

(F) RT-PCR expression of il10 in melatonin-treated Tr1 cells with or without the addition of UO126. Data are representative of five independent experiments (means and SEM). *$p < 0.05$ of unpaired t test versus vehicle and signaling inhibitor control condition. **$p < 0.05$ versus vehicle of UO126-treated condition.

(G) Flow cytometry analysis of IL-10 expression as in (C), in wild-type mice and ROR-α-deficient mice.

(H) ROR-α putative binding site present in the il10 promoter (lower panel) and chromatin immunoprecipitation with anti-ROR-α (upper panel). Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of unpaired t test.

(I) Luciferase activity of HEK293 cells transfected with a luciferase reporter construct for the il10 promoter. Data are representative of three independent experiments (means and SEM). *$p < 0.05$ of unpaired t test.

(J) Schematic diagram depicting the effects of melatonin in Tr1 cells.

and was used as template for real-time PCR. All primers and probes were provided by Applied Biosystems and were used on the ViiA 7 Real-Time PCR System (Applied Biosystems). Expression was normalized to the expression of the housekeeping gene Gapdh.

Immunoblot Analysis

For immunoblot analysis, cells were lysed with radio-immunoprecipitation buffer supplemented with protease inhibitor "cocktail" (Sigma-Aldrich). Total lysates of the different T cell subsets (40 μg) were resolved by electrophoresis through 4%–12% Bis-Tris Nupage gels (Invitrogen) and were transferred onto PVDF membranes (Millipore). The following primary antibodies were used: anti-ROR-α (Abcam), anti-MTNR1A (Santa Cruz), anti-total and phospho-Erk1/2 (Cell Signaling), anti-total C/EBP α (Cell Signaling), anti-phospho C/EBPα (Cell Signaling), anti-Nfil3 (Santa Cruz), and anti-GADPH (Abcam). Blots were developed with SuperSignal West Femto Maximum Sensitivity Substrate as suggested by the manufacturer (Pierce).

Statistical Analysis

A Poisson regression model was used to assess the impact of season 6-SM levels and the number of clinical relapses, generating an incidence rate ratio (IRR) and corresponding 95% confidence intervals (CI). A repeated-measures mixed model was used to assess the effect of treatment and its interaction with time in EAE experiments. A linear regression model was used to analyze the relationship between serum melatonin levels and IL-17 or IL-10 gene expression. Differences between two or more conditions were analyzed with Student's t test, Mann-Whitney test, one-way ANOVA, or Wilcoxon rank-sum test when appropriate. p values <0.05 were considered significant. Unless otherwise specified, all data are presented as mean ± SEM. All statistical analyses were performed using Stata v12 (Statacorp).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.08.025.

AUTHOR CONTRIBUTIONS

M.F.F. performed epidemiologic analyses and in vitro and in vivo experiments, analyzed data, and wrote the manuscript. I.D.M. and S.P.M. performed in vitro and in vivo experiments with a comparable contribution. A.Y. performed in vitro and in vivo experiments. M.E.B. participated in human sample recollection, data analysis, and interpretation. L.G. and M.G. performed human in vitro experiments. B.P. performed bioinformatics analysis. M.C.Y. participated in human sample recollection, data analysis, and interpretation. C.Z. provided knockout mice. V.K.K. and G.A.R. edited the manuscript and participated in data interpretation. M.F.F., F.J.Q., and J.C. interpreted data, conceived and supervised the study, and edited the manuscript.

ACKNOWLEDGMENTS

This study was supported by the Allende Foundation and the MSIF Du-Preé grant (to M.F.F.), a grant from Biogen Idec and Novartis Argentina (to M.F.F. and J.C.) and Merck Serono Argentina (to J.C.), AI093903 and NS087867 from the NIH, RG4111A1 and JF2161-A-5 from the National Multiple Sclerosis Society, and PA0069 from the International Progressive MS Alliance to F.J.Q. We would like to thank Drs. Lora Hooper, Bart Staels, Vincent Laudent, Mitch Lazar, and Daniel Tenen for generously providing reagents used in these studies. We would also like to thank Jessica Kenison-White for technical assistance with mice colonies.

Received: December 4, 2014
Revised: May 4, 2015
Accepted: July 8, 2015
Published: September 10, 2015

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