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Melatonin Protects CD4+ T Cells from Activation-Induced Cell Death by Blocking NFAT-Mediated CD95 Ligand Upregulation

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Over the past 20 y, the hormone melatonin was found to be produced in extrapineal sites, including cells of the immune system. Despite the increasing data regarding the biological effects of melatonin on the regulation of the immune system, the effect of this molecule on T cell survival remains largely unknown. Activation-induced cell death plays a critical role in the maintenance of the homeostasis of the immune system by eliminating self-reactive or chronically stimulated T cells. Because activated T cells not only synthesize melatonin but also respond to it, we investigated whether melatonin could modulate activation-induced cell death. We found that melatonin protects human and murine CD4+ T cells from apoptosis by inhibiting CD95 ligand mRNA and protein upregulation in response to TCR/CD3 stimulation. This inhibition is a result of the interference with calmodulin/calcineurin activation of NFAT that prevents the translocation of NFAT to the nucleus. Accordingly, melatonin has no effect on T cells transfected with a constitutively active form of NFAT capable of migrating to the nucleus and transactivating target genes in the absence of calcineurin activity. Our results revealed a novel biochemical pathway that regulates the expression of CD95 ligand and potentially other downstream targets of NFAT activation. *The Journal of Immunology*, 2010, 184: 3487–3494.

T he highly evolutionarily conserved hormone melatonin (*N*-acetyl-5-methoxytryptamine) was first isolated from the bovine pineal gland (1) and subsequently described in algae, protozoa, plants, and a variety of mammals (2). In mammals, melatonin is recognized as a major regulator of seasonal and circadian rhythms by means of its daily nocturnal increase in plasma levels (3). In addition, melatonin presents antioxidant, anti-inflammatory, and immunomodulatory activities (4–6).

Over the past two decades, a substantial body of evidence has demonstrated that melatonin can be synthesized by a number of nonendocrine extrapineal sites, including gut, retina, skin, and the bone marrow (7–9). More recently, it has been shown that stimulated human lymphocytes have the necessary machinery to produce and release large amounts of melatonin (10). Along with the fact that melatonin receptors are present in neutrophils (11), monocytes (12), and lymphocytes (13), it was proposed that melatonin exerts intracrine, autocrine, and paracrine immuno-modulatory activities (10).

Indeed, several studies have reported the effect of exogenous melatonin on lymphoproliferation (14), activation (15), differentiation to effector Th1 cells (16), migration (17), and cytokine production by human lymphocytes (18, 19). In particular, endogenous production of melatonin by human lymphocytes has been related to enhanced release of IL-2 and upregulation of IL-2R (20), suggesting that melatonin may be involved in the clonal expansion of Ag-stimulated human T lymphocytes. Importantly, the effects of melatonin on activated T cell death remain largely unknown.

Activation-induced cell death (AICD), an apoptotic process that occurs after restimulation of T cells via their Ag receptor complex (TCR/CD3), was initially described in T cell hybridomas (21) and later on demonstrated in all major T cell subsets, including Th1, Th2, and Th17 (22, 23). AICD is dependent on the upregulation of Fas ligand (CD95L), which binds to its cognate receptor Fas (CD95), triggering the activation of a caspase-dependent apoptotic pathway (24).

AICD is involved in the maintenance of the T cell homeostasis, at least, in three different ways. First, AICD participates in the deletion of self-reactive T cell clones, avoiding the accumulation of potentially dangerous cells (25). Second, it contributes to the elimination of chronically stimulated cells (26), which can produce harmful levels of cytokines. Finally, AICD is partially responsible for the elimination of an expanded and no longer necessary T cell population after chronic infection (27). In fact, mice or humans who present defects in the CD95/CD95L pathway show accumulation

of activated T cells with lymphadenopathy and splenomegaly and generally develop severe autoimmunity (28, 29).

In this study, we found that melatonin protects human and murine T cells from AICD by inhibiting anti-CD3–mediated CD95L upregulation. Melatonin prevented NFAT dephosphorylation induced by TCR/CD3 stimulation, thereby interfering with NFAT translocation to the nucleus. Melatonin also blocked the transactivation of the human CD95L reporter promoter, even when cells were cotransfected with an NFAT construct. Importantly, melatonin failed to inhibit the transactivation of either NFAT-responsive promoter or the human CD95L promoter in cells transfected with a constitutively active form of NFAT. Taken together, our data demonstrated for the first time a role for melatonin in T cell survival by preventing TCR/CD3-mediated NFAT activation of CD95L transcription and subsequent AICD.

Materials and Methods

Mice, cell lines, and reagents

Spleen and lymph nodes of 6- to 8-wk-old BALB/c mice were used as a T lymphocyte source. Human PBMCs were obtained from healthy donors after written consent. Jurkat cells and DO11.10 T lymphocyte hybridoma were a gift of Dr. Douglas Green (Saint Jude Research Children's Hospital, Memphis, TN). Primary cells were grown at 37°C in 5% CO_2 in DMEM supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium piruvate, 100 mM nonessential amino acids, 100 mM vitamins, 10 mM 2-ME, 100 mg/ml streptomycin, and 100 U/ml penicillin. Cell lines were regularly maintained at 37°C in 5% CO_2 in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin.

Melatonin, Percoll, and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO). Cyclosporine A (CsA) was obtained from LC Laboratories (Woburn, MA). PMA was obtained from Calbiochem (San Diego, CA), and Ficoll-Paque Plus was from GE Healthcare (Chalfont St. Giles, U.K.). RPMI 1640, DMEM, L-glutamine, penicillin, streptomycin, FCS, and PHA, nonessential amino acids, vitamins, and 2-ME were purchased from Life Technologies (Rockville, MD). Ionomycin was obtained from Calbiochem, and anti-CD3 (clone 2C11), anti-CD95L (clone MFL3), and anti-CD14 Abs were purchased from BD Pharmingen (San Diego, CA). Anti–NF-κB and anti–phospho-NF-κB Abs were bought from Cell Signaling Technology (Beverly, MA). Anti-NFAT1 Abs (clone 67.1) were kindly provided by Dr. Anjana Rao (Harvard Medical School, Boston, MA).

Generation of murine T cell blasts

Spleens or lymph nodes were removed aseptically from BALB/c mice and teased into single-cell suspension. RBCs were lysed with ammonium chloride solution, and adherent cells were removed by 2-h incubation in tissue-culture plates. For generation of T cell blasts, 1×10^6 cells were stimulated for 48 h with 1 mg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 Abs in flat-bottomed six-well plates in a final volume of 1 ml. Cells were washed and cultured for 4 more d with 100 U/ml recombinant human IL-2 (proleukin, Zodiac Produtos Farmacêuticos, São Paulo, Brazil). Later, dead cells and debris were eliminated by centrifugation over Ficoll-Paque Plus.

Generation of T cell blasts from human PBMCs

PBMCs were purified from healthy donors by Ficoll-Paque density centrifugation. Lymphocytes were isolated from PBMCs by an additional Percoll gradient. In all experiments, no less than 75% of the recovered cells were CD3-positive, and no more than 2% were CD14-positive, as assessed by flow cytometry (data not shown). T cell blasts were generated by stimulating 2×10^6 cells/ml with 1 μg/ml PHA for 16 h, followed by additional 6-d culture in the presence of 30 U/ml recombinant human IL-2, added every 2 d of culture. Percentage of $CD3^+$ T cells was at least 98% in all experiments (data not shown).

Apoptosis induction

DO11.10 hybridoma, murine, and human T cell blasts were induced to apoptosis by different stimuli, including 1 μg/ml plate-bound anti-CD3 Abs, 10 ng/ml PMA plus 1 μM ionomycin, 1 μg/ml soluble anti-Fas Abs, 10 μM Teniposide (VM-26), and 1 μM actinomycin D. In some cases,

during these death-inducing stimuli, cells were treated with different concentrations of melatonin, lactacystin, CsA, or N-acetylcysteine.

Assessment of apoptosis and cell death

Apoptosis was estimated by multiple parameters (30). For cell cycle analysis of total DNA content, cells were collected, lysed, and nuclei stained with hypotonic fluorescent solution (50 μg/ml PI, 0.1% Triton X-100, and 0.1% sodium citrate). Samples were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA), and numbers represent the mean percentage of cells with DNA fragmentation ± SD in triplicate samples. Cell death was also quantified by PI exclusion. Cells were stained with 10 μg/ml PI and analyzed by flow cytometry. Numbers represent the mean percentage of PI-positive cells ± SD in triplicate samples. Some samples were also analyzed morphologically or based in changes of light scattering properties of the dead cells (31).

RT-PCR and quantitative PCR

Total mRNA were extracted by TRIzol (Invitrogen, Carlsbad, CA) from DO11.10 cells and converted to cDNA using Superscript III (Invitrogen) as described by the manufacturer's protocols.

Quantitative PCR was performed using TAQMAN technology (Applied Biosystems, Foster City, CA). CD95L mRNA expression was analyzed by the Gene Expression Assay catalog number Mm00438864_m1, CD95 by Mm01204974_m1, and cellular FLICE-like inhibitory protein (c-FLIP) by Mm01255578_m1, and results were normalized by GAPDH expression, assayed by the Endogenous Controls Assay catalog number 4352932E. The expression values are relative to the amplification of unstimulated cells.

Quantification of CD95L protein expression

CD95L protein expression was detected by flow cytometry as previously described (32) using anti-CD95L.PE Abs (clone MFL3; BD Pharmingen). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

SDS-PAGE and Western blot

Protein samples were resolved as previously described (33). Cells were harvested, washed in ice-cold PBS, lysed directly in SDS sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 2.5% 2-ME), and boiled for 5 min. Samples were resolved under reducing conditions in SDS-polyacrylamide gels. Separated proteins were then blotted onto polyvinylidene difluoride membranes and probed with an appropriate dilution of primary Abs. Reactions were detected with suitable secondary Ab conjugated to HRP (GE Healthcare) using ECL solution (Pierce, Rockford, IL).

Intracellular localization of NFAT

For intracellular localization of NFAT1, protein cells were attached to cover slips previously coated for 1 h with 2% gelatin and left unstimulated or were stimulated for 16 h at 37°C with 1 μM ionomycin. Fifteen minutes prior to stimulation, 1 mM melatonin or 10 mM CsA was added to cells. Subsequently, cells were fixed in 3% paraformaldehyde, permeabilized with 0.1% Nonidet P-40, and stained with anti-NFAT1.PE Abs and 300 mM DAPI. The cells were photographed under ×100 magnification with a Zeiss Axiovert S100 microscope (Zeiss, Oberkochen, Germany), using red and blue filters. MERGE represents the overlay of red- and blue-filter images.

Promoter transactivation assays

Jurkat cells (3×10^6/600 μl) were electroporated (950 μF, 250 V) in a 0.4-cm Gene Pulser Cuvette (Bio-Rad, Hercules, CA) with GenePulser II electroporator (Bio-Rad). Cells were cotransfected with 4 μg luciferase reporter plasmids [either 3xNFAT/AP-1.luciferase (p3xNFAT), which was constructed by fusion of three distal NFAT element-binding sequences of the IL-2 promoter (34), or hFasLpromoter.luciferase (pHFLP), a plasmid that contains the luciferase gene under the control of a 1.2-kb region of human CD95L promoter (35)] and 0.4 μg RL.TK Renilla luciferase plasmid (pRL-TK), which encodes the Renilla luciferase and is used for normalization of transfection efficiency. In some experiments, a third plasmid (40 μg) was cotransfected: pcDNA5 (empty vector), pcDNA5. NFAT1 or pL.IRES2.NFAT1 (wild-type NFAT1), or pL.IRES2.CA-NFAT1 (constitutively active form of NFAT). Plasmid constructions are described in detail elsewhere (36, 37). After 24 h, cells were washed and stimulated at 37°C for 6 h with 10 nM PMA plus 1 μM ionomycin in the presence or absence of 1 mM melatonin or 10 mM CsA. The next day, cells were harvested and lysed for 15 min at room temperature with 50 μl of 1× passive lysis buffer (Promega, Madison, WI). Crude extracts (10 μl) were

analyzed in a Veritas TM Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) using Dual-Luciferase Reporter Assay System (Promega). Luciferase activities were expressed as relative light units.
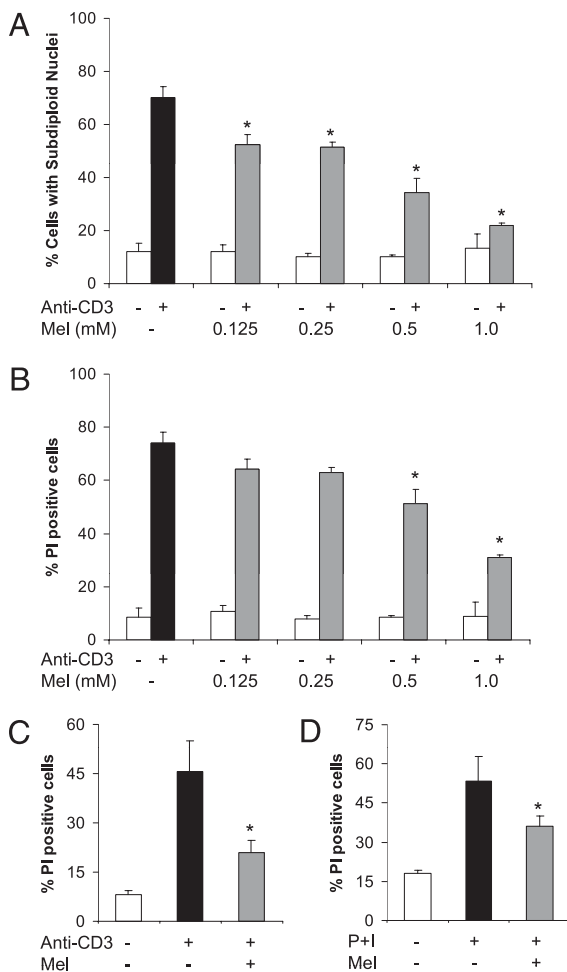
Statistical analysis

Experiments were always performed in triplicate and at least three times. Data are presented as mean values ± SD. Statistical analysis of the data was performed using one-way ANOVA and Tukey as a posttest. Differences between experimental groups were considered significant for $p \leq 0.01$ or, in some cases, $p \leq 0.05$. All statistic tests were performed using Prism version 5 software (GraphPad, San Diego, CA).

Results

Melatonin protects T cells from anti-CD3–induced apoptosis

To evaluate the influence of melatonin on T cell survival, DO11.10 hybridoma cells were stimulated with immobilized anti-CD3 Abs to mimic anti-CD3–induced apoptosis (AICD). Different concentrations of melatonin (0.125–1 mM) were added to anti-CD3–stimulated cells, and, after 18 h of culture, apoptosis was evaluated by cell cycle analysis of DNA content. As shown in Fig. 1A,
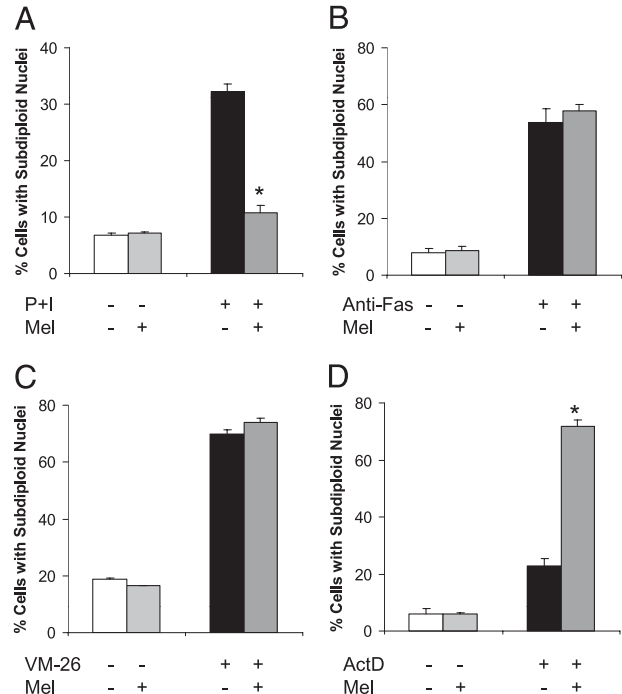
melatonin inhibited the appearance of subdiploid cells in a dose-dependent manner, reaching almost full protection at the highest dose (1 mM). To exclude the possibility that melatonin, rather than protecting cells from AICD, was converting anti-CD3–induced apoptosis into a necrotic form of cell death, analysis of PI exclusion was also performed. Our data showed that melatonin prevented the appearance of PI-positive cells (Fig. 1B). Importantly, melatonin was not toxic to cells in any concentration used (Fig. 1A, 1B), and the vehicle had no influence on the results (data not shown).

Next, we tested whether melatonin could also protect primary T lymphocytes from AICD. As naive cells are not susceptible to AICD, we generated AICD-sensitive murine T cell blasts in vitro and stimulated these cells with plate-bound anti-CD3 Abs. Melatonin significantly inhibited AICD in murine T cell blasts either derived from spleen (Fig. 1C) or lymph nodes (data not shown). In addition, freshly isolated human T cells obtained from PBMCs were sensitized to AICD by 6-d culture with PHA and IL-2 and killed by incubating with PMA plus ionomycin, in the presence or absence of 1 mM melatonin (Fig. 1D). Again, melatonin was able to significantly protect human T cell blasts from AICD. Altogether, these results demonstrated that melatonin is able to protect human and mouse T lymphocytes from AICD.

Effect of melatonin on cell death is specific for TCR/CD3-mediated apoptosis

To test the specificity of the antiapoptotic effect of melatonin, DO11.10 cells were incubated for 18 h with different apoptogenic stimuli. Interestingly, melatonin suppressed apoptosis induced by the combination of PMA and ionomycin, which activates the same

FIGURE 1. Effect of melatonin on AICD of T cells. A and B, DO11.10 cells were stimulated with or without plate-bound anti-CD3 Abs for 18 h in the presence of different concentrations of melatonin. C, Murine splenic T cell blasts were stimulated with or without plate-bound anti-CD3 Abs for 18 h in the presence or not of 1mM melatonin. D, T cell blasts derived from human peripheral blood leukocytes were stimulated with 10 ng/ml PMA plus 1 μM ionomycin (P+I) in the presence or not of 1 mM melatonin. A, Apoptosis was estimated by cell cycle analysis. Numbers represent the average percentage ± SD of cells with subdiploid DNA content. B–D, Cell death was quantified by PI exclusion. Each set of experiments was performed at least three times. *$p \leq 0.01$.

FIGURE 2. Melatonin protects DO11.10 cells from PMA/ionomycin-induced death but not from other apoptosis-inducing stimuli. DO11.10 cells were stimulated for 18 h with 10 ng/ml PMA plus 1 μM ionomycin (P+I) (A), 1 μg/ml anti-Fas Abs (clone Jo2) (B), 10 μM Teniposide (VM-26) (C), or actinomycin D (ActD) (D) and simultaneously treated or not with 1 mM melatonin. Apoptosis was estimated by cell cycle analysis. Numbers represent the average percentage ± SD of cells with subdiploid DNA content. Figure shows representative data of three independent experiments. *$p \leq 0.01$.
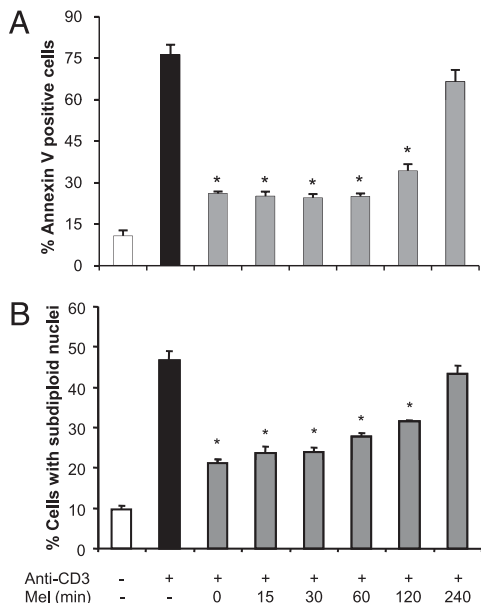
FIGURE 3. The addition of melatonin up to 2 h after anti-CD3 stimulation protects DO11.10 cells from AICD. DO11.10 cells were stimulated for 18 h with plate-bound anti-CD3 Abs, and melatonin was added after different periods, as indicated in the graphic. Apoptosis was estimated by Annexin V-FITC staining (*A*) and cell cycle analysis (*B*). Numbers represent the average percentage ± SD of cells stained for Annexin V-FITC or with subdiploid DNA content. Figure shows representative data of two independent experiments. *Statistically different from anti-CD3 stimulation, $p \leq 0.01$.

tine sulfate, or UVC irradiation (data not shown). Additionally, melatonin synergized with actinomycin D, increasing its proapoptotic effect (Fig. 2*D*). These data suggest that melatonin specifically prevented TCR/CD3-induced apoptosis and that this protection is upstream of CD95 engagement.

Melatonin inhibits anti-CD3–induced CD95L upregulation, an early event of AICD induction

To evaluate whether the melatonin was involved in blocking early or late events of AICD, we stimulated DO11.10 with immobilized anti-CD3 Abs and added melatonin after different time points (0–240 min). Fig. 3 shows that melatonin only protected T cells from death if added up to 120 min after the initiation of anti-CD3 stimulation, suggesting that this molecule acts on early events of AICD.

Because CD95 and CD95L expression is augmented in T cells after TCR restimulation (24, 38) and the sensitivity to AICD is associated with a reduction in the levels of the caspase-8 inhibitor c-FLIP (39), we examined whether melatonin could alter the expression of one or more of these genes. When DO11.10 cells were stimulated for 4 h with immobilized anti-CD3 Abs in the presence or absence of 1 mM melatonin, we found no effect of melatonin on CD95 and c-FLIP gene expression (Fig. 4*A*). However, melatonin dramatically suppressed the anti-CD3–mediated upregulation of CD95L at both the mRNA (Fig. 4*A*) and protein (Fig. 4*B*) levels.

Melatonin blocks NFAT activation

The transcription factor NFAT is a major modulator of CD95L expression. Therefore, we investigated whether the inhibitory effect of melatonin on CD95L was mediated by interference on the NFAT-dependent pathway. Because the first step of NFAT activation is its dephosphorylation by calcineurin (40), we analyzed the

pathways as the TCR/CD3 signaling (Fig. 2*A*), but did not protect DO11.10 cells from stimulation with 1 μg/ml agonistic anti-CD95 Abs (Fig. 2*B*), teniposide (VM-26) (Fig. 2*C*), etoposide, vincris-

FIGURE 4. Melatonin inhibits anti-CD3–induced CD95L expression in DO11.10. *A*, Total mRNA was extracted from DO11.10 cells stimulated with anti-CD3 Abs for 4 h in the presence or absence of 1 mM melatonin and converted to cDNA. Then, quantitative PCR was performed for CD95, FLIP, and CD95L. The expression was normalized by GAPDH expression of each sample. *B*, CD95L protein expression in DO11.10 cells stimulated or not with anti-CD3 in the presence of 1 mM melatonin for 8 h. The expression was measured by flow cytometry using anti–CD95L-PE Abs.
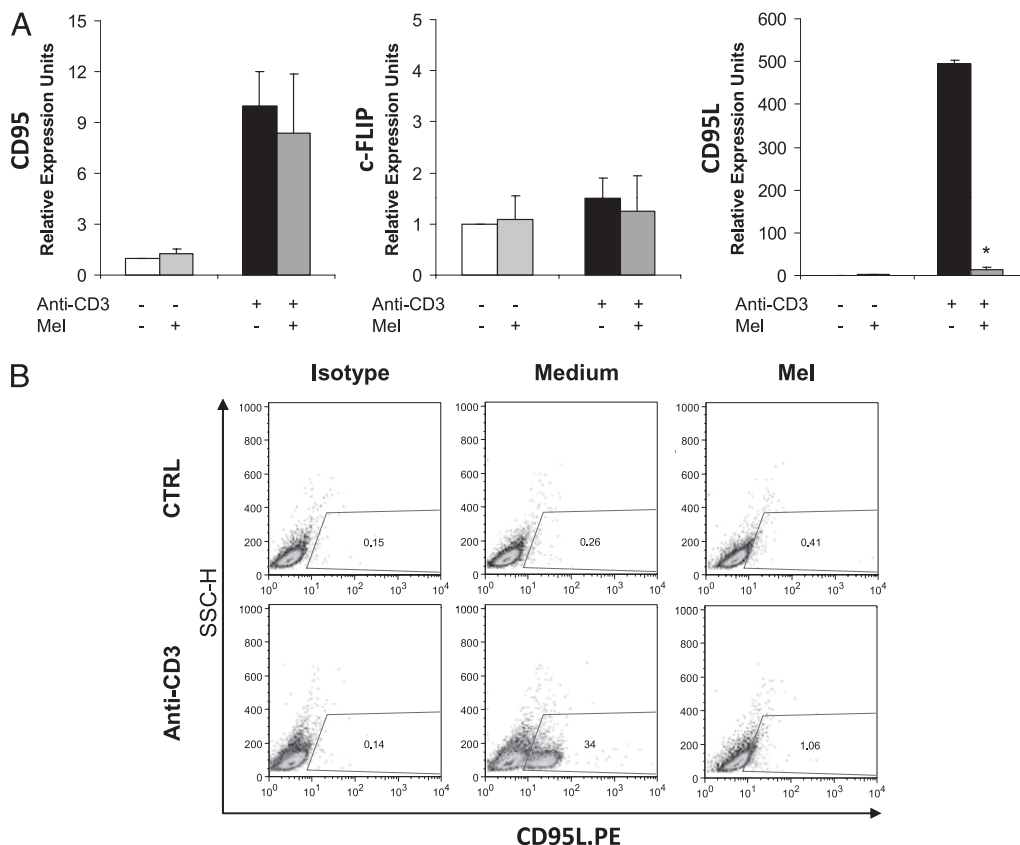
effect of melatonin on anti-CD3–induced dephosphorylation of NFAT. As illustrated in Fig. 5A, anti-CD3 stimulation augmented the proportion of dephosphorylated (active)/phosphorylated (inactive) forms of NFAT1, which was dose dependently inhibited by melatonin. This effect was accompanied by a melatonin-induced retention of NFAT1 in the cytosol, as seen by fluorescence microscopy of DO11.10 cells stimulated with anti-CD3 (Fig. 5B). The same results were found when we used ionomycin, a Ca^{2+} ionophore that activates NFAT1 and induces its translocation to the nucleus without activating other anti-CD3–induced pathways (Supplemental Fig. 1).

Using luciferase reporter assays, we confirmed that melatonin interferes with NFAT activation. Jurkat T cells transfected with p3xNFAT reporter plasmid, combined or not with pcDNA5.NFAT1 or the pcDNA5 vector control, and stimulated with PMA plus ionomycin produced different levels of luciferase activity (Fig. 5C, 5D). In every situation, the addition of melatonin resulted in lower luciferase expression (Fig. 5C, 5D). Importantly, melatonin was unable to interfere with PMA plus ionomycin-induced luciferase activity when the p3xNFAT reporter plasmid was cotransfected with the pL.IRES2.CA-NFAT1, containing a constitutively activated form of NFAT1 (Fig. 5E). Finally, we observed a similar effect of melatonin when Jurkat cells were transfected with a reporter plasmid construct containing the luciferase gene under the control of the human CD95L promoter (Fig. 5F).

Discussion

The effect of melatonin on cell death varies depending on the cell type and the nature of stimuli. According to the literature, melatonin can protect cells from death, can be innocuous, or can even potentiate apoptosis (reviewed in Ref. 41). For instance, injection of melatonin enhances thymic cellularity (42), whereas pinealectomy seems to accelerate thymus involution and consequently lower the cellular immune response (43, 44). In the case of mature T lymphocytes, exogenous melatonin was able to diminish the cytotoxicity of x-ray irradiation (45) or idarubicin (46). However, in some cases, instead of providing protection, melatonin potentiates T cell death, as demonstrated in the case of CD95-induced death in Jurkat cells cultured in glutathione-depleted medium (47). Our own results show that whereas melatonin protects from stimulation with anti-CD3 Abs or with the combination of PMA and ionomycin, it has no effect on anti-CD95 or teniposide-induced cell death and potentiates apoptosis triggered by actinomycin D.

It is well known that TCR/CD3 stimulation results in rapid CD95L upregulation, and CD95L-mediated CD95 oligomerization is believed to be the primary mechanism of AICD (24, 38, 48). We observed that melatonin has no effect on direct CD95 stimulation with the agonistic Ab, suggesting that melatonin prevented AICD prior to CD95 engagement. In fact, melatonin was not able to prevent AICD when added to DO11.10 cells after 2 h of anti-CD3 stimulation, indicating that melatonin blocked early events of TCR/CD3-induced apoptosis.

FIGURE 5. Melatonin blocks NFAT activation, which is essential for AICD in DO11.10 cells. *A*, Western blot for NFAT1. The Ab used detects both the phosphorylated/inactive and nonphosphorylated/active form of NFAT1. Cell extracts were obtained after 1 h of anti-CD3 stimulation in the presence or absence of different concentrations of melatonin. β-actin detection was used as a loading control. *B*, Immunofluorescence for NFAT1. DO11.10 cells were stimulated for 1 h with anti-CD3 Abs in the presence or absence of 1 mM melatonin and then stained with DAPI and anti–NFAT1-PE Abs. *C–F*, Jurkat cells were transfected by eletroporation with 0.4 μg pRL-TK and 4 μg p3xNFAT (*C–E*) or 0.4 μg pRL-TK and 4 μg pHFLP (*F*). In addition, Jurkat cells were transfected with 40 μg pcDNA5.Vector or pcDNA5.NFAT1 (*D*) or pL.IRES2.NFAT1 or pL.IRES2.CA-NFAT1 (*E*, *F*). Cells were stimulated or not with 10 ng/ml PMA plus 1 μM ionomycin (P+I) in the presence of 1 mM melatonin or 10 mM CsA, and after 6 h, total cell lysates were obtained. Luciferase activity was measured as described and expressed as relative light units relative to the treatment presented in the first graphic column. *$p \leq 0.05$.
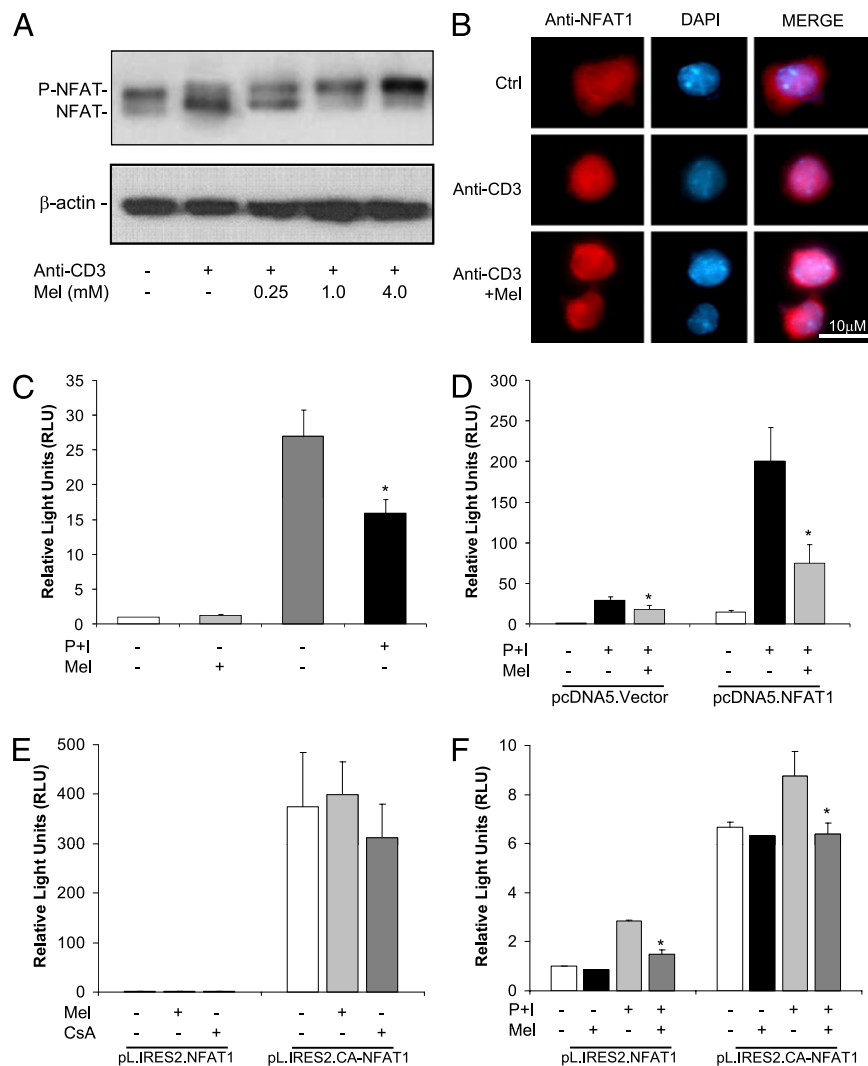
TCR/CD3 stimulation can be mimicked by the combination of PMA and ionomycin, which can trigger similar biochemical pathways but independently and downstream of TCR/CD3 clustering, activation of Src-family kinases, phosphorylation of ITAMs, and recruitment of specific adaptor or scaffold proteins. As melatonin also blocked PMA/ionomycin-induced apoptosis, it is unlikely that the protection involves interference with one or more of these very early events of TCR/CD3 activation.

Melatonin was shown to bind to calmodulin in a Ca^{2+}-dependent and melatonin receptor-independent way (49, 50). One important target of calmodulin is calcineurin, a phosphatase responsible for the dephosphorylation and activation of the transcription factor NFAT. Notably, NFAT1 is the most abundant NFAT family member in T lymphocytes (51) and a major transactivator of the CD95L promoter (52, 53). NFAT1 is strongly activated after TCR/CD3 stimulation, and the involvement of NFAT1 on CD95L-induced T cells apoptosis is evidenced by studies using CsA, a specific inhibitor of calcineurin-mediated dephosphorylation of NFAT that abolishes CD95L expression and drastically reduces AICD in thymocytes and T cells (52, 54). Although some downstream targets of calmodulin were already shown to be inhibited by melatonin, such as Ca^{2+}/calmodulin-dependent kinase II (55) and calmodulin-dependent phosphodiesterase (56), there are no data in the literature so far implicating melatonin as a modulator of NFAT activation. In this study, we demonstrated for the first time that melatonin prevented, in a dose-dependent fashion, both anti-CD3– and ionomycin-induced dephosphorylation of NFAT1 and its translocation to the nucleus. Using the p3xNFAT or the pHFLP reporter plasmids, we confirmed that melatonin is capable of preventing promoter transactivation induced by the combination of PMA and ionomycin. In addition, melatonin was able to suppress the enhancement of promoter activity induced by enforced expression of an NFAT1 construct. Importantly, transfection of CA-NFAT1 in Jurkat cells induced basal activity of a p3xNFAT promoter that was not blocked by addition of either melatonin or CsA. Noteworthy, CA-NFAT1 was generated by mutations from serine to alanine in the regulatory domain of an NFAT1 molecule that maintains the nuclear localization signal exposed, thereby allowing the protein to migrate to the nucleus even in the absence of calcineurin activity (57). Altogether, our data indicate that melatonin acts upstream of NFAT migration to the nucleus, specifically avoiding NFAT1 dephosphorylation by Ca^{2+}/calmodulin-dependent calcineurin. Interestingly, the fact that melatonin receptor-deficient mice did not show any overt T cell deficiency supports our hypothesis that the effect of melatonin on FasL expression and consequent T cell survival is mediated through direct binding of melatonin to calmodulin and consequent inhibition of NFAT activation.

Because of their weak interaction (58), it was proposed that the binding of melatonin to calmodulin would not be possible at the physiological level of melatonin found in circulation (59, 60). Nevertheless, it is important to consider that melatonin is present at much higher levels in some microenvironments—in particular, at inflammatory sites (61). Indeed, inhibition of calmodulin by melatonin was suggested to be involved in many physiological processes, including microtubule polymerization (62), rat myotube-acethylcoline receptor expression (63), neuronal NO synthase expression (64), estrogen receptor α activation (65), and cytoskeleton rearrangement (66) and also to avoid the progression of scoliosis in mice and humans (67, 68).

The prevention of anti-CD3–mediated CD95L upregulation by melatonin reduces AICD levels in T cells, which may have an important role for the survival of T cell populations and the development and extension of T cell-mediated immune responses.

Interestingly, melatonin was shown to increase Ab titers to thymus-dependent Ags and is being tested as a vaccine adjuvant against bacterial and viral infection (69, 70). Also, the death of T cells throughout the Ag-driven clonal expansion in vivo is proportional to the Ag concentration (71) and is counteracted by the presence of TLR ligands through an unknown mechanism independent of Bim, Bcl-2, and Bcl-x_L (72). As TLR stimulation leads to a potent proinflammatory response and melatonin level is increased during inflammation, it is reasonable to conceive that melatonin-mediated downregulation of CD95L may play a role in the survival of T cells during an immune response.

Interference with expression of CD95L in T lymphocytes may also have an impact on the survival of APCs during the initiation or maintenance of the immune response. OTII transgenic $CD4^+$ T cells stimulated by OVA-pulsed dendritic cells (DCs) express CD95L and are able to kill OVA-pulsed DCs, but not bystander DCs (73). Danger signals, including TLR ligands, inhibit CD95L expression in $CD4^+$ T cells by upregulating costimulatory molecules (74). In addition, TLR stimulation induces the release of PGE_2 by APCs, which in turn prevents TCR/CD3-mediated increase in CD95L and killing of target cells (32).

In light of these new findings, future studies are necessary to investigate if long-term usage of melatonin, such as in patients with sleep disorders and jet-lagged flight crew members, has any effects on T cell survival and development of autoimmune disorders. Indeed, some studies had already shown a correlation between melatonin administration and worsening of collagen-induced arthritis (75) and the development of experimental autoimmune encephalomyelitis (76). On the other hand, melatonin could be useful for treatment of diseases that develop with T cell loss, such as HIV/AIDS. In fact, HIV^+ patients with reduced serum melatonin levels presented a more accelerated progression of the disease (77), and in a phase II pilot clinical study, s.c. treatment with IL-2, in combination with melatonin, enhanced $CD4^+$ T cell count in HIV^+ patients (78).

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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