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IL-10-induced STAT3/NF-κB crosstalk modulates pineal and extra-pineal melatonin synthesis

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Abstract

Immune-pineal axis activation is part of the assembly of immune responses. Proinflammatory cytokines inhibit the pineal synthesis of melatonin while inducing it in macrophages by mechanisms dependent on nuclear factor-κB (NF-κB) activation. Cytokines activating the Janus kinase/signal transducer and activator of transcription (STAT) pathways, such as interferon-gamma (IFN-γ) and interleukin-10 (IL-10), modulate melatonin synthesis in the pineal, bone marrow (BM), and spleen. The stimulatory effect of IFN-γ upon the pineal gland depends on STAT1/NF-κB interaction, but the mechanisms controlling IL-10 effects on melatonin synthesis remain unclear. Here, we evaluated the role of STAT3 and NF-κB activation by IL-10 upon the melatonin synthesis of rats' pineal gland, BM, spleen, and peritoneal cells. The results show that IL-10-induced interaction of (p)STAT3 with specific NF-κB dimmers leads to different cell effects. IL-10 increases the pineal's acetylserotonin O-methyltransferase (ASMT), *N*-acetylserotonin, and melatonin content via nuclear translocation of NF-κB/STAT3. In BM, the nuclear translocation of STAT3/p65-NF-κB complexes increases ASMT expression and melatonin content. Increased pSTAT3/p65-NF-κB nuclear translocation in the spleen enhances phosphorylated serotonin *N*-acetyltransferase ((p)SNAT) expression and melatonin content. Conversely, in peritoneal cells, IL-10 leads to NF-κB p50/p50 inhibitory dimmer nuclear translocation, decreasing (p)SNAT expression and melatonin content. In conclusion, IL-10's effects on melatonin production depend on the NF-κB subunits interacting with (p)STAT3. Thus, variations of IL-10 levels and downstream pathways during immune responses might be critical regulatory factors adjusting pineal and extra-pineal synthesis of melatonin.

KEYWORDS
bone marrow, IL-10, immune-pineal axis, NF-κB, spleen and peritoneal cells, (p)STAT3

1 | INTRODUCTION

Pineal melatonin is produced rhythmically by pinealocytes in vertebrates,[1–4] reaching its peak levels during the night phase and synchronizing the organism to the environmental light/dark cycle.[5] Melatonin is also produced by extra-pineal sources such as the gastrointestinal tract, bone marrow (BM), spleen, thymus, and immunocompetent cells, with several autocrine and paracrine functions.[5,6] The concept of an immune-pineal axis (IPA) was proposed by Markus et al. in 2007 describing a transient shift from pineal to local production of melatonin during the different phases of inflammatory processes.[5] This physiopathologic pattern of regulation upon the melatonergic system was observed in humans[7–9] and animal models.[10–13] The disruption of melatonin production is also associated with several pathologies, such as several neurodegenerative diseases, severe pain, diabetes type 2, and cancer.[14–16] Therefore, determining the molecular mechanisms controlling melatonin synthesis is relevant for a better understanding of the processes regulated by this indolamine in health, physiopathologic, and pathologic conditions.

Circadian regulation of pineal gland melatonin synthesis relies upon the sympathetic-induced rhythm in the expression and activity of the phosphorylated serotonin *N*-acetyltransferase (pSNAT).[1] This enzyme converts serotonin into *N*-acetylserotonin (NAS) which is then methylated by the acetylserotonin *O*-methyltransferase (ASMT) action forming melatonin. Several transcriptional factors might participate in melatonin synthesis and, among them, the nuclear factor κB (NF-κB)[17] has a central role in integrating melatonergic and immunological systems. NF-κB family has five members: RelA (p65), RelB, cRel, p50 (its precursor is p105), and p52 (its precursor is p100). The subunits p65, RelB, and cRel have the transactivation domain (TAD^+) that induces RNA transcription. NF-κB-TAD^+ dimers are associated with the increase of melatonin in the pineal gland (p65/p65 and p50/p65 NF-κB dimers) and phagocytes (p50/p65 and cRel/p65 NF-κB dimers) by increasing *Snat* transcription.[4,18–21] In contrast, TAD negative (TAD^-) NF-κB dimers (mainly p50/p50 NF-κB dimers) decrease *Snat* transcription.[18,19,21,22]

Pathogen-associated molecular patterns (e.g., lipopolysaccharides [LPS]) and proinflammatory cytokines (e.g., tumor necrosis factor [TNF]) activate the TAD^- p50/p50 NF-κB dimers inhibiting *Snat* transcription and pineal melatonin production.[5] Considering the inhibitory effects of melatonin upon adhesion molecules,[23,24] the reduction of circulating nocturnal melatonin allows cellular migration through the endothelial layer at night. Conversely, at the injury site, LPS and TNF induce the activation of TAD^+ p65/cRel NF-κB dimers in phagocytes, increasing local melatonin synthesis.[4,5] In the resolution phase of the inflammatory process, melatonin produced by macrophages enhances phagocytosis,[25] while, in the pineal gland, the inhibition of TAD^- NF-κB by glucocorticoids (GCs) leads to the restoration of melatonin synthesis.[4] Other immune regulators like interferon-gamma (IFN-γ) modulate pineal melatonin synthesis. This cytokine activates the signal transducer and activator of transcription 1 (STAT1) and TAD^+ NF-κB crosstalk, enhancing *Snat* gene transcription and melatonin production.[21]

We previously showed that interleukin-10 (IL-10), a cytokine with pleiotropic effects in immunoregulation and inflammation, controls melatonin synthesis by modulating pSNAT and ASMT expression in immune cells from the BM and spleen.[26] Considering that STAT1 interacts with NF-κB and potentiates pineal melatonin synthesis,[21] that IL-10 activates STAT3 signaling, and that STAT3/NF-κB cytoplasmatic interactions can lead to NF-κB activation,[27] the present work aimed to test whether and how IL-10-induced STAT3/NF-κB crosstalk regulates melatonin synthesis in the pineal gland and immune cells of different tissues. We chose to evaluate immune cells from a primary lymphoid organ associated with cell production, the BM, cells from the peritoneal cavity, which would be firstly activated during a local immune response, and cells from the spleen, an organ implicated in immune surveillance and immunomodulation.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

All of the animal protocols were performed following the ethical standards of the National Council on Experimental Animal Control. The Ethical Committee for Animal Experimentation was approved by license numbers 207/2014 and 253/2016 at the Institute of Bioscience of the University of São Paulo.

2.2 | Animals

Male Wistar rats (8–12 weeks old, 250–300 g), receiving water and food ad libitum, were kept at 22.0 ± 2.0°C under a 12:12 h light/dark cycle (lights on at 06:00 h = Zeitgeber time zero or ZT0). Animals were obtained from the animal facility of the Department of Physiology, Institute of Bioscience of the University of São Paulo (IB-USP, São Paulo, Brazil).

2.3 | Experimental design

Animals were killed by decapitation at 10 ZTs (0.25, 3, 6, 9, 11.75, 12.25, 15, 18, 21, and 23.75) and the pineal gland, peritoneal cells, BM of the femurs, and spleen were collected. We decided to use only male animals to avoid estrous cycle interference with melatonin production by the pineal gland and immune-competent cells.

2.4 | Drugs and reagents

Rat IL-10 (I9154), Cucurbitacin I (C4493), and PDTC (ammonium pyrrolidinedithiocarbamate, P8765) were purchased from Sigma. RPMI-1640 and DMEM medium were purchased from GIBCO. Penicillin/streptomycin was purchased from Life Technologies.

2.5 | Measurement of IL-10 and IL-10R expressions

IL-10 and IL-10R expressions throughout the day were determined by real-time quantitative PCR (RT-qPCR) for glands and by flow cytometry for BM, spleen, and peritoneal cells.

2.6 | BM, splenic, and peritoneal cell sampling and processing

BM cells were obtained from the femur by flushing with RPMI-1640 medium or phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4), supplemented with 100 U/mL penicillin and 0.1 mg/mL streptomycin (RPMI-PS, PBS-PS). Splenic cells were obtained by mechanical dispersion using a 100 μm cell strainer filter (Falcon®), and red blood cells were then lysed with ice-cold lysis buffer (0.15 M $ClNH_4$, 10 mM $NaHCO_3$, and 0.1 mM EDTA, 2 mL, 4°C, 5 min) and washed with RPMI-PS. Peritoneal cells were obtained by washing the peritoneal cavity with ice-cold PBS-PS. After centrifugation (500 g, 10 min), cells were resuspended in RPMI-PS or PBS.

2.7 | In vitro treatments

Cells were plated on a 24-multiwell plate (2×10^6 cells/well, 500 μL) and maintained at 37°C, 5% CO_2. After 2 h, cells were stimulated with a STAT3 phosphorylation inhibitor (Cucurbitacin I, 3 μM), an NF-κB inhibitor (PDTC, 3 μM), both compounds, or the respective vehicles for 30 min, and then incubated with IL-10 (3 or 100 ng/mL). The cells were maintained in culture for 3 or 6 h, depending on the protocol. The cells (3 h incubation with IL-10) were used to measure protein expressions by flow cytometry. The supernatants (6 h incubation with IL-10) were collected for melatonin quantification by ELISA.

2.8 | Flow cytometry

Cells were fixed with 4% paraformaldehyde (in PBS, 10 min at 4°C), permeabilized with 0.1% Triton X-100 (in PBS, 10 min, room temperature), blocked with 3% bovine serum albumin (1 h at 25°C), incubated with primary antibodies (overnight at 4°C), and labeled with secondary conjugated antibodies (1 h at 25°C) (Table 1). Samples were acquired with an Amnis® FlowSight® (Luminex), and data were analyzed using IDEAS® software.

TABLE 1 Antibodies information.

Proteins target	Primary antibodies	Secondary antibodies
IL-10 and IL-10 receptor	Mouse anti-IL-10Rβ (sc271969) or rabbit anti-IL-10 (ab9969)	Donkey anti-mouse FITC-conjugated IgG (Invitrogen A24501), donkey anti-rabbit PE-conjugated IgG (BioLegend), donkey anti-goat PECy7-conjugated IgG (sc3859)
Melatonergic enzymes	Rabbit anti- SNAT (S0564), pSNAT (S0814) or ASMT (IM-044)	
STAT3 and NF-κB	Mouse anti-STAT3 (Cell Signaling 9130) and rabbit anti-p50 (ab7971), p65 (ab7970) or cRel (sc-70X)	
pSTAT3 and NF-κB	Rabbit anti-pSTAT3 (ab30647), goat anti-p50 (sc1190) and mouse anti-p65 (ab13594)	
Nuclear staining	–	Propidium iodide (Molecular Probes P3566)

2.9 | Image analysis using IDEAS® software

The analyses and their description were obtained from the instructions in the IDEAS User's Manual Guide (luminexcorp.com/download/amnis-ideas-software-user-manual/). The gating strategy to identify the nuclear localization of (p)STAT3 or NF-κB and the (p)STAT3/NF-κB colocalization is shown in Figure S1.

2.9.1 | STAT3, pSTAT3, and NF-κB nuclear localization:

The nuclear localization is measured using the Similarity feature: the log-transformed Pearson's correlation coefficient, which measures the degree to which two images are linearly correlated within a masked region. A mask is the set of pixels that contains the region of interest, which, in our case, is the cell nucleus marked with propidium iodide.

2.9.2 | STAT3/NF-κB and pSTAT3/NF-κB colocalization

(p)STAT3/NF-κB co-localization was measured using the Bright Detail Similarity R3 Feature. This feature is designed to compare the small bright image detail of two images and can be used to quantify the co-localization of two probes in a defined region. The Bright Detail Similarity R3 feature is the log-transformed Pearson's correlation coefficient of the localized bright spots with a radius of 3 pixels or less within the masked area in the two input images. Since the bright spots in the two images are either correlated (in the exact spatial location) or uncorrelated (in different spatial locations), the correlation coefficient varies between 0 (uncorrelated) and 1 (perfect correlation) and does not assume negative values. The coefficient is log-transformed to increase the dynamic range between zero and infinite. Generally, a Similarity Bright Detail value of 3.0 indicates a high degree of correlation, and an object with a value lower than 1.0 has no significant similarity. The Similarity feature was also applied to determine whether the (p)STAT3/NF-κB colocalization occurs in the nucleus.

2.10 | Pineal gland culture

Rat pineal glands were incubated at 37°C, 95% O_2, 5% CO_2 in BGJb medium, supplied with PS, 2 mM glutamine, and 0.1 mg/mL ascorbic acid (1 gland per well, 200 μL) for 48 h before treatment to obtain an utterly denervated preparation, as previously described.[28]

After 48 h, cultured glands were placed in fresh medium for 1 h and stimulated or not with 3 μM Cucurbitacin I and/or 3 μM PDTC for 30 min, then IL-10 (3 ng/mL) for 30 min and finally noradrenaline (NA; 10 nM) for 90 min or 5 h, depending on the protocol. Supernatants (5 h) were collected for melatonin and NAS quantification by HPLC, and the glands (90 min) were collected for mRNAs quantification by RT-qPCR or to measure nuclear NF-κB accumulation by electrophoretic mobility shift assay.

2.11 | RT-qPCR

Total RNA was extracted using TRIzol, and the RNAs were reverse-transcribed to form complementary DNAs (cDNA) by SuperScript™ III Reverse Transcriptase, following the manufacturer's instructions. The qPCR was performed with 1 μL cDNA, 10 nM of each corresponding pair of primers (Table 2), and SYBR Green (Applied Biosystems) in a final volume of 15 μL. Relative quantification was calculated by the $2^{-\Delta\Delta C_t}$ formula, where $\Delta\Delta C_t$ represents the cycle difference normalized to *β-actin* for *Il-10*, *Il-10rα*, and *Il-10rβ* expressions or normalized to *18S* rRNA for *Snat*, *14-3-3* and *Asmt* expressions.

2.12 | Electrophoretic mobility shift assay

Nuclear protein extraction of the pineal glands was conducted according to a protocol previously described

TABLE 2 Primer sequences.

Gene target	Primer sequences
Il-10	F: 5′-CCTCTGGATACAGCTGCGAC-3′
	R: 5′-TGAGTGTCACGTAGGCTTCT-3′
Il-10rα	F: 5′-GATGTTGCCGCGTTTACTCC-3′
	R: 5′-TCCGTACTTCTTGAGGGCCA-3′
Il-10rβ	F: 5′-CTGGTGCCAGCTCTAGGAATG-3′
	R: 5′-GCGGTCTTGGAAGTACCAGT-3′
Snat	F: 5′-AGCGCGAAGCCTTTATCTCA-3′
	R: 5′-AAGTGCCGGATC TCATCCAA-3′
Asmt	F: 5′-AGCGCCTGCTGTTCATGAG-3′
	R: 5′-GGAAGCGTGAGAGGTCAAAGG-3′
18S	F: 5′-CGGCTACCACATCCAAGGAA-3′
	R: 5′-GCTGGAATTACCGCGGCT-3′

by Ferreira et al.[22] NF-κB nuclear translocation was performed using double-stranded consensus probes (5′AGTTGAGGGGACTTTCCCAGGC-3′) labeled with γ-ATP-^{32}P. The optical density of formed bands was quantified using the software program ImageJ and normalized by the unstimulated group.

2.13 | Melatonin quantification by ELISA in immune cells

Due to the sensitivity of the method and the amount of melatonin produced in culture by the immune cells, melatonin present in the medium was measured using ELISA kits following the manufacturer's instructions (Immuno Biological Laboratories). The detection limits of the melatonin ELISA kits were 0.5 pg/mL.

2.14 | Melatonin and NAS quantification by HPLC in pineal glands

Melatonin and NAS contents in the medium of cultured pineal glands were determined by HPLC using an electrochemical detection system, as previously described.[28]

2.15 | Bioinformatic searches

The different protein–protein interaction networks between IL-10, STAT3, and NF-κB subunits were visualized by STRING[29,30] (Figure S2). In silico analysis of the binding sites for NF-κB and STAT3 transcription factors were recognized by scanning a position weight matrix (PWM) against consensus sequence flanking 5′- regions of *Snat*/*SNAT* (GenBank- NC_051345.1 from *Rattus norvegicus* and NC_000017.11 from *Homo sapiens*) and *Asmt*/*ASMT* promoters (GenBank- NC_051347.1 from *Rattus norvegicus* and NC_000023.11 from *Homo sapiens*), using JASPAR 2020[32] and the tool TFsearch. NF-κB and STAT3 PWMs visualization and *Snat*/*SNAT* and *Asmt*/*ASMT* genes are shown in the supplementary material (Figure S3; Table S1).

2.16 | Statistical analysis

Data were expressed as mean ± SEM. Time series were analyzed using the Cosinor analysis (Cosinor online app[32]). Comparisons were performed using Student "*t*" (two samples comparison) or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for treatments with different concentrations of IL-10. Two-way ANOVA with multiple comparisons was used to analyze treatments with IL-10 and inhibitors. $p < .05$ were considered statistically significant. Analyses were performed using GraphPad Prism 7.0.

3 | RESULTS

We first evaluated whether the expression of *Il-10* mRNA or the alpha (*Il-10ra*) and beta (*Il-10rb*) subunits of the IL-10 receptor showed daily variations in the pineal gland. Besides the absence of rhythms tested by COSINOR analysis, both *Il-10* (Figure 1A) and its receptor (Figure 1B) presented higher expression during the middle of the light phase. Thus, the subsequent experiments were carried out at ZT06, the predictable moment with higher responsiveness of pineal glands to IL-10, the lower presence of circulating melatonin, and with immune cells presenting the lower expression of melatonergic enzymes.[26]

Previously, we demonstrated that IL-10 at different concentrations could modulate melatonin synthesis in BM and spleen cells.[26] To determine the concentration with the maximum effect, pineal glands were stimulated with IL-10 at different concentrations. IL-10 at 3 ng/mL enhanced *Asmt* mRNA levels, an effect significantly reverted by 100 ng/mL of IL-10 (Figure 1C). Besides an increased tendency, the treatments did not affect *Snat* mRNA levels (Figure 1C). These effects led to an increase in NAS and melatonin at 3 ng/mL of IL-10 and an increase in NAS levels at 30 ng/mL of IL-10, but interestingly, a higher concentration (100 ng/mL) had no significant effect on NAS and melatonin synthesis (Figure 1D). Thus, IL-10 has a dual effect on *Asmt* mRNA expression, NAS, and melatonin levels but not *Snat* mRNA expression.

Recent studies from our laboratory have shown that transcription factors of the STAT family can colocalize with NF-κB in the *SNAT* promoter region and thus regulate melatonin synthesis.[21] IL-10 binding to its receptor triggers the STAT3 signaling pathway. Using in silico promoter analysis, we found that, in addition to the protein-protein interaction between STAT3 and NF-κB family members (Figure S2), STAT3 and NF-κB responsive elements colocalize in *Snat*/*SNAT* and *Asmt*/*ASMT* rat (Figure 2A) and human (Figure 2B) regulatory regions of the genes. Thus, we evaluate whether the effect of IL-10 on melatonin synthesis depends on the cross-stimulation of the STAT3 and NF-κB pathways. Our results showed that IL-10 enhanced NF-κB nuclear translocation and DNA binding in the pineal gland (Figure 1E), and the increment of melatonin levels by

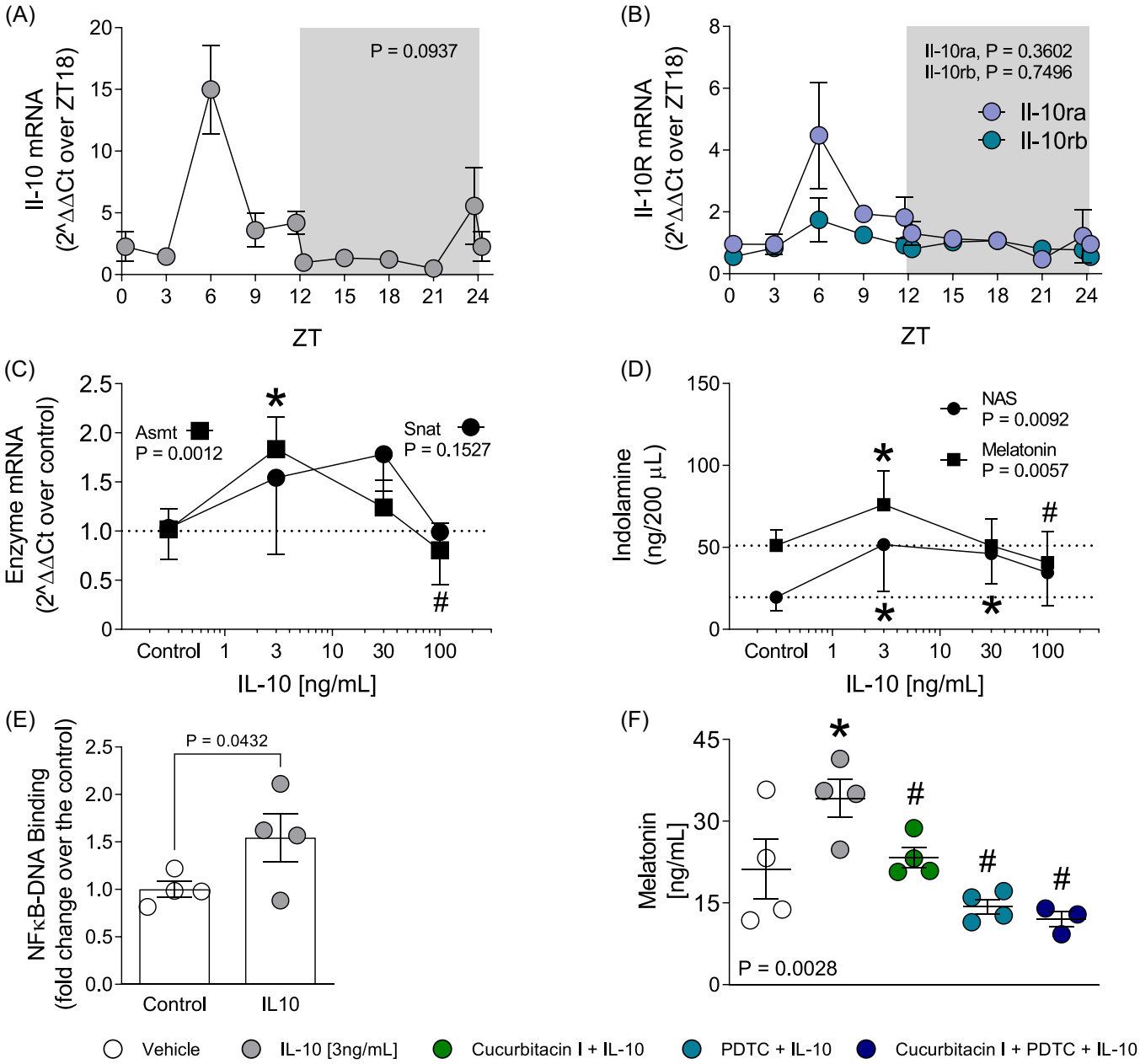

FIGURE 1 IL-10 effects on melatonin synthesis in the pineal gland. (A) IL-10 and (B) IL-10ra and IL-10rb mRNA expressions throughout the day. ZT0 is defined as the moment when lights go on, and ZT12 is when the lights are turned off. Results are expressed as the mean ± SEM ($n = 4$ animals per point). Data were analyzed using the Cosinor method. The gray background marks the dark period. (C) SNAT and Asmt mRNA expressions of cultured pineal glands and (D) melatonin and NAS (*N*-acetylserotonin) medium levels. Cultured pineal glands were pre-incubated with vehicle or IL-10 (3, 30, or 100 ng/mL, for 30 min) and stimulated with NA (10 nM for an additional 5 h). Data are presented as mean ± SEM. ($N = 4$–11 glands per point) and were analyzed by one-way ANOVA with Tukey's post hoc. *$p < .05$ vs. control, #$p < .05$ vs. IL-10 (3 ng/mL). (E) NF-κB-DNA binding activity of nuclear extracts of pineal glands incubated with vehicle or IL-10 (3 or 100 ng/mL, for 30 min). Data are expressed as fold-change over the control of NF-κB-DNA-binding activity of nuclear extracts from the control group. (F) NA-induced melatonin levels after inhibiting STAT3 (Cucurbitacin I, 3 µM) and NF-κB (PDTC, 3 µM) pathways. Data are presented as mean ± SEM ($N = 3$–4 glands per point) and were analyzed by two-way ANOVA with Tukey's post hoc. *$p < .05$ vs. control.

IL-10 was reversed by PDTC blockage of NF-κB activation or cucurbitacin I inhibition of STAT3 phosphorylation (Figure 1F).

We then evaluated the expressions of IL-10 and IL-10-β receptor subunit (IL-10RB) throughout the day on BM (Figure 3A,B), peritoneal cavity cells (Figure 3C,D), and splenic cells (Figure 3E,F). Similar to the observed in pineal glands, the expression of IL-10RB did not show a daily variation in BM cells (Figure 3B) and peritoneal cells (Figure 3D).

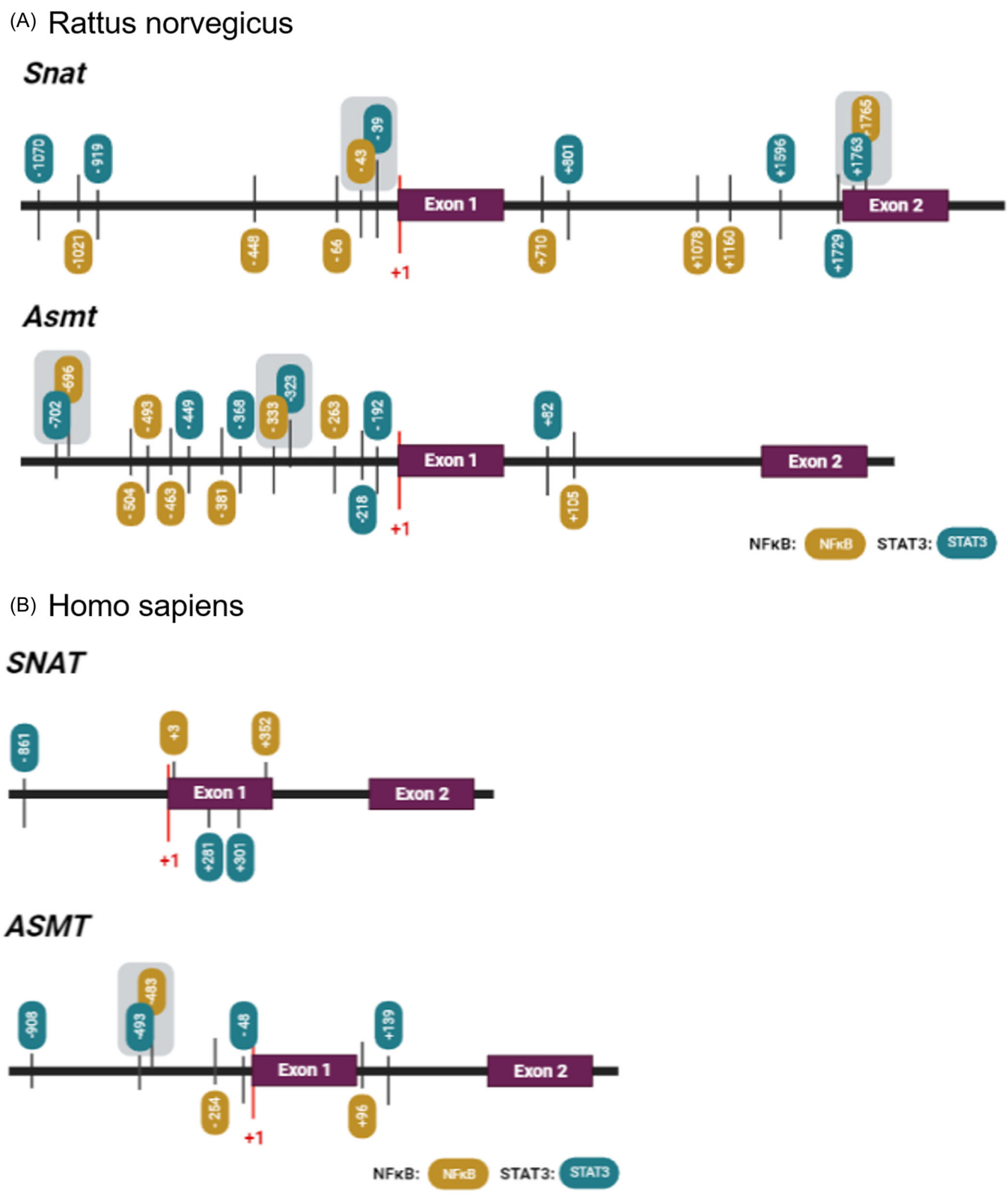

FIGURE 2 STAT3/NF-κB interaction in rats (A) and humans (B). Responsive elements of the *Snat*/*SNAT* and *Asmt*/*ASMT* genes to the transcription factors STAT3 and NF-κB. Created with BioRender.com.

Curiously, the expression of IL-10RB presented a daily variation in the spleen cells, with higher levels in the middle of the light phase (Figure 3F). IL-10 did not present significant rhythms among the groups.

IL-10 induced a tissue-dependent effect on the melatonergic system. At the concentration of 3 ng/mL, IL-10 increased in 50% the ASMT expression (Figure 4A) and melatonin levels (Figure 4B) in BM cells. In splenic cells, low-dose IL-10 treatment increased SNAT and

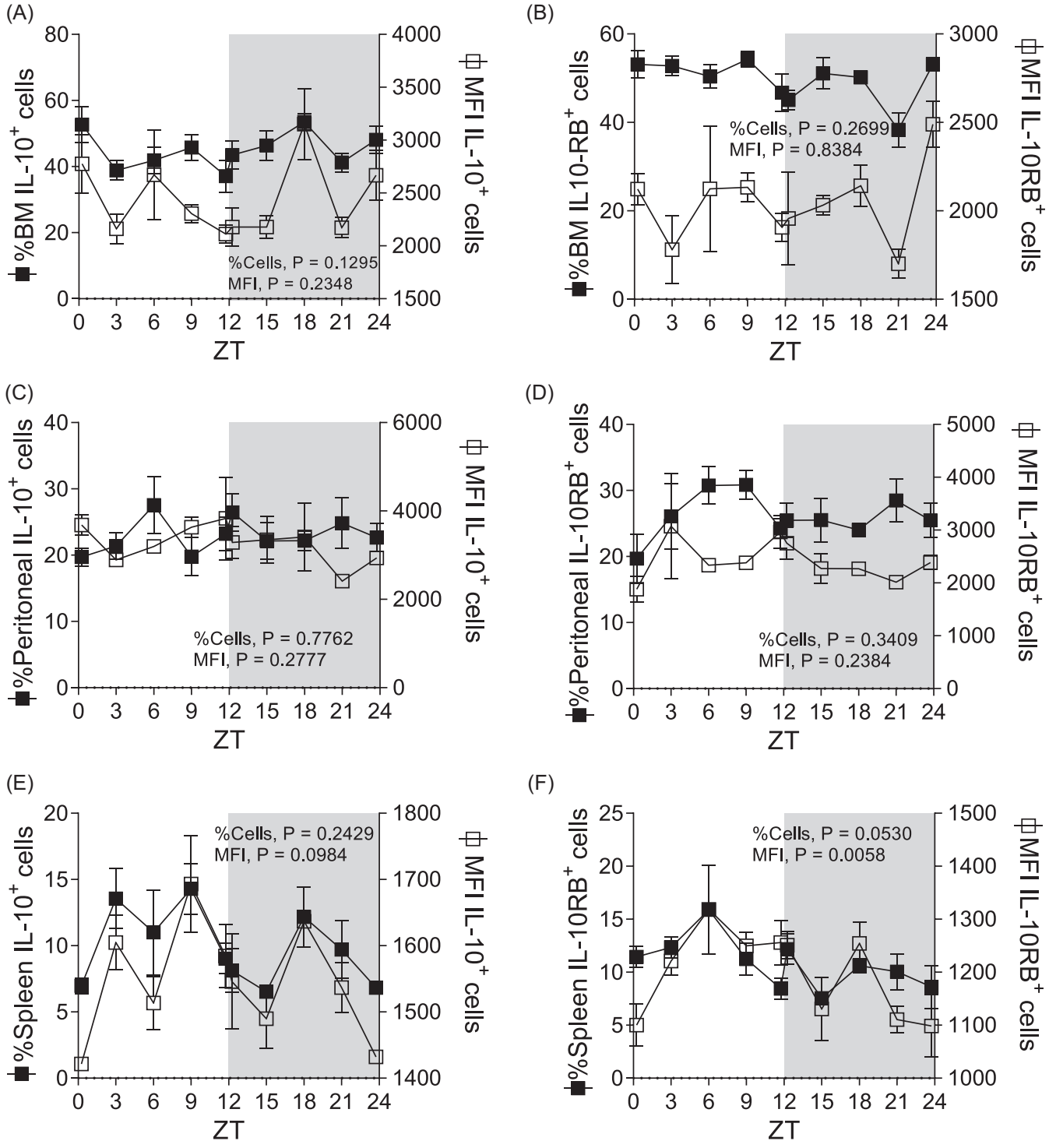

FIGURE 3 IL-10 and IL-10R daily variation (A and B) BM, (C and D) peritoneal, and (E and F) splenic cells. Results are expressed as the mean ± SEM of % of cells and MIF values ($n = 4$ animals per point). Data were analyzed using Cosinor rhythm determination. The gray background marks the dark period.

pSNAT expressions (Figure 4E) resulting in a proportional 50% increase in melatonin synthesis (Figure 4F). Conversely, in peritoneal cells, IL-10 decreased pSNAT (Figure 4C) and melatonin levels (Figure 4D). On the other hand, IL-10 at 100 ng/mL decreased melatonin levels in the cells obtained from BM (Figure 4A), peritoneal cavity (Figure 4C), and spleen (Figure 4E). The reductions in melatonin production were strongly associated with the proportional 40%–50% reductions in ASMT expressions compared to the respective control groups (Figure 4B,D,F). Interestingly, in spleen cells the 100 ng/mL IL-10-induced melatonin reduction (Figure 4F) occurred even with a sustained increase of pSNAT expression (Figure 4E), reinforcing the role of ASMT as the rate-limiting enzyme of the melatonin synthesis pathway.[33]

To determine whether the effects of IL-10 observed in BM, peritoneal, and spleen cells are also regulated by the

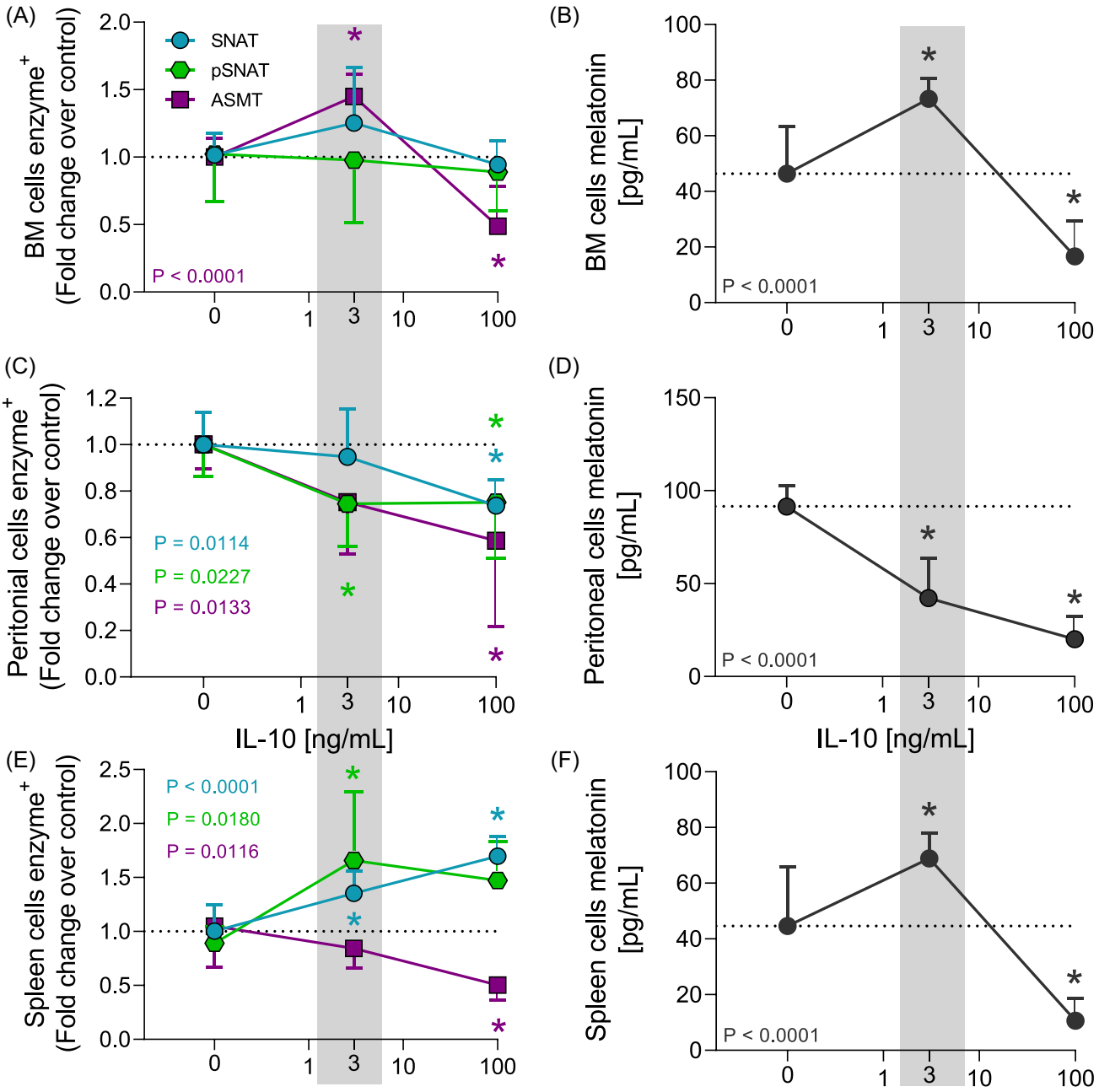

FIGURE 4 IL-10 effects on the melatonergic system in BM (A and B), peritoneal (C and D), and splenic cells (E and F). Cellular melatonergic biosynthetic enzymes' expressions (A, C, and E) and culture medium melatonin levels (B, D, and F) after stimulus with IL-10 (3 and 100 ng/mL) for 6 h. Results are expressed as mean ± SEM, $n = 6–9$ animals from two independent experiments. Data were analyzed by one-way ANOVA with Tukey's post hoc.

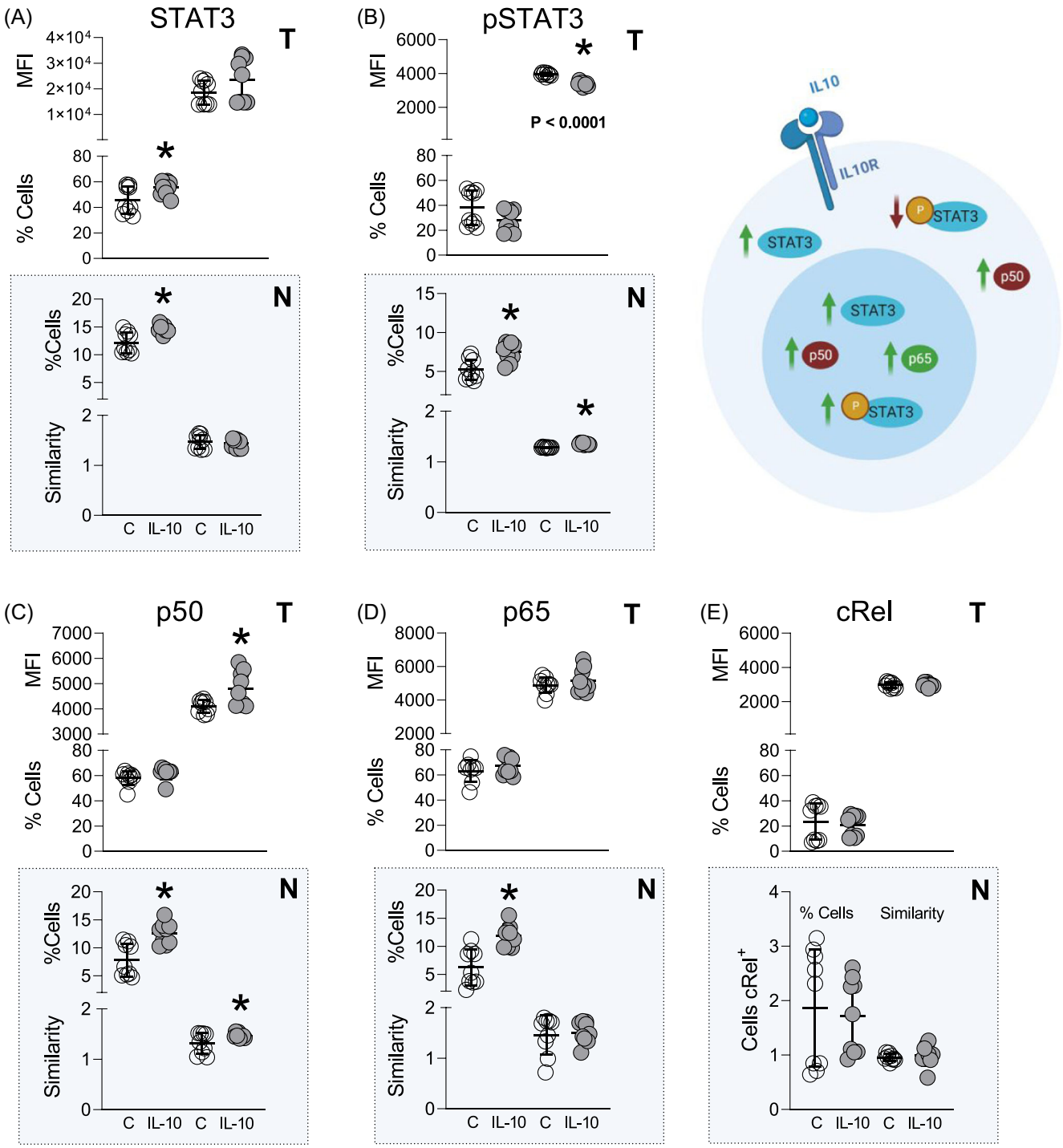

FIGURE 5 IL-10 modulation in BM cells. Total (T) and nuclear (N—blue boxes) expression of STAT3 (A), pSTAT3 (B), p50 (C), p65 (D), and cRel (E) NF-κB subunits of BM cells treated (IL-10) or not (control group, C) with IL-10 3 ng/mL. Data are presented as % of cells expressing the transcriptional factors and as MIF (total cell) or similarity (Nucleus) representing expression levels. Results are expressed as mean ± SEM, $n = 8$–10 animals from two independent experiments. Comparisons performed by Student "*t*" test. *$p < .05$ vs. control. The inserted scheme summarizes the main findings (created with BioRender.com).

crosstalk of the STAT3 and NF-κB pathways, we then evaluate the total expressions and the nuclear localization of STAT3, pSTAT3, and NF-κB (p50, p65, and cRel) subunits, as well as the variations on STAT3/NF-κB and pSTAT3/NF-κB colocalizations. For those experiments, we used the 3 ng/mL IL-10 concentration, as it imposed different effects upon the melatonergic enzymes depending on the origin of the cells (gray bars in Figure 4).

In BM cells (Figures 5 and 6), IL-10 increased the expressions of STAT3 (Figure 5A), pSTAT3 (Figure 5B),

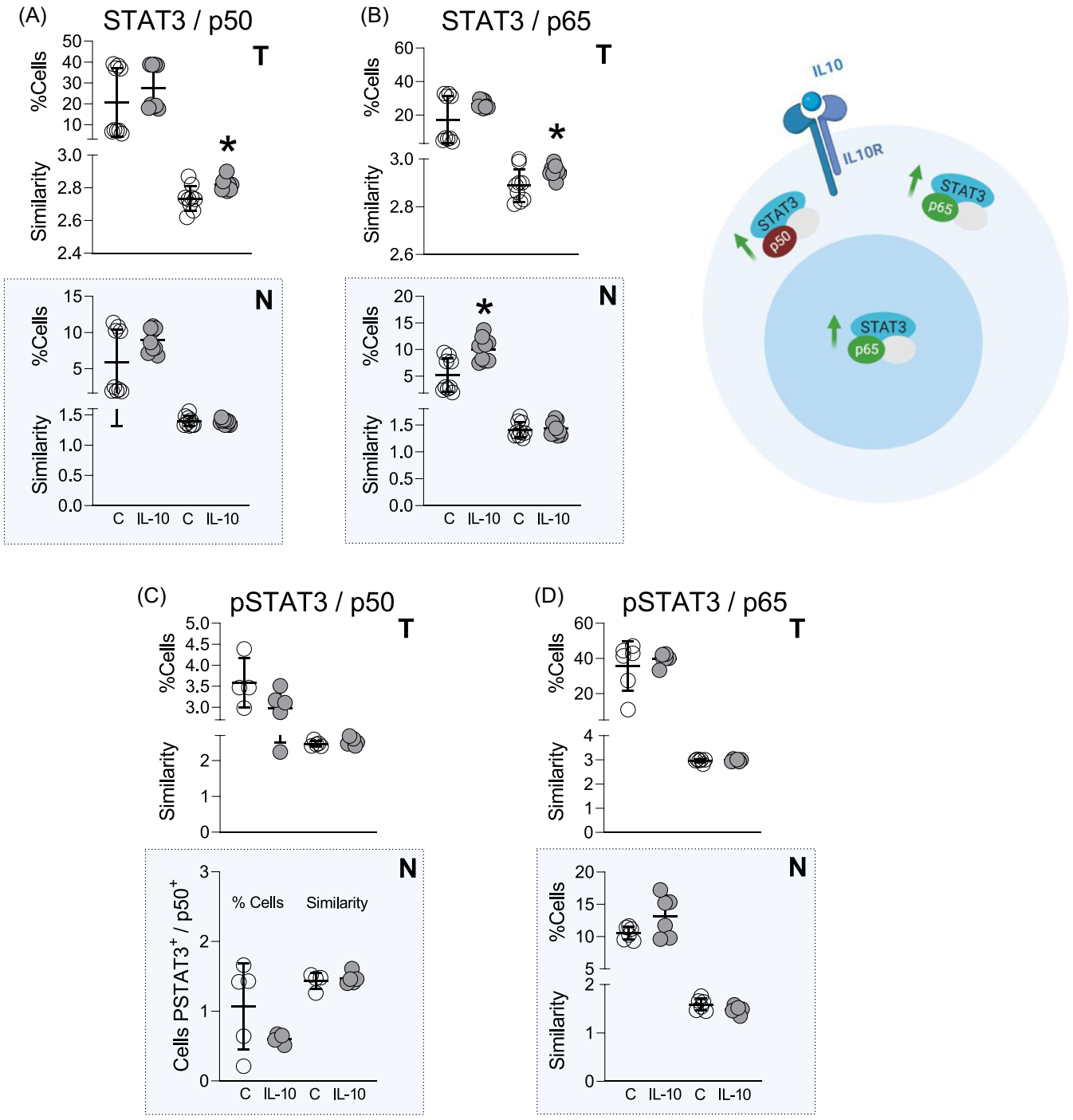

FIGURE 6 IL-10 modulation in BM cells. Total (T) and nuclear (N—blue boxes) colocalizations of STAT3/p50 (A), STAT3/p65 (B), pSTAT3/p50 (C), and pSTAT3/p65 (D) of BM cells treated (IL-10) or not (control group, C) with IL-10 3 ng/mL. Data are presented as % of cells expressing the colocalized transcriptional factors and as similarity scores representing expression levels. Results are expressed as mean ± SEM, $n = 8–10$ animals from two independent experiments. Comparisons performed by Student "t" test. *$p < .05$ vs. control. The inserted scheme summarizes the main findings (created with BioRender.com).

and P50 (Figure 5C), and the STAT3 colocalization with p50 (Figure 6A) and P65 (Figure 6B). The nuclear localization of STAT3 (Figure 5A), pSTAT3 (Figure 5B), and P50 (Figure 5C), as well as the nuclear colocalization of STAT3/p65 (Figure 6B), were also increased after IL-10 stimulus. The expression and nuclear localization of cRel, pSTAT3/p50, and pSTAT3/p65 colocalization did not change (Figures 5 and 6).

Cells from the peritoneal cavity (Figures 7 and 8) stimulated with IL-10 presented an increase in the

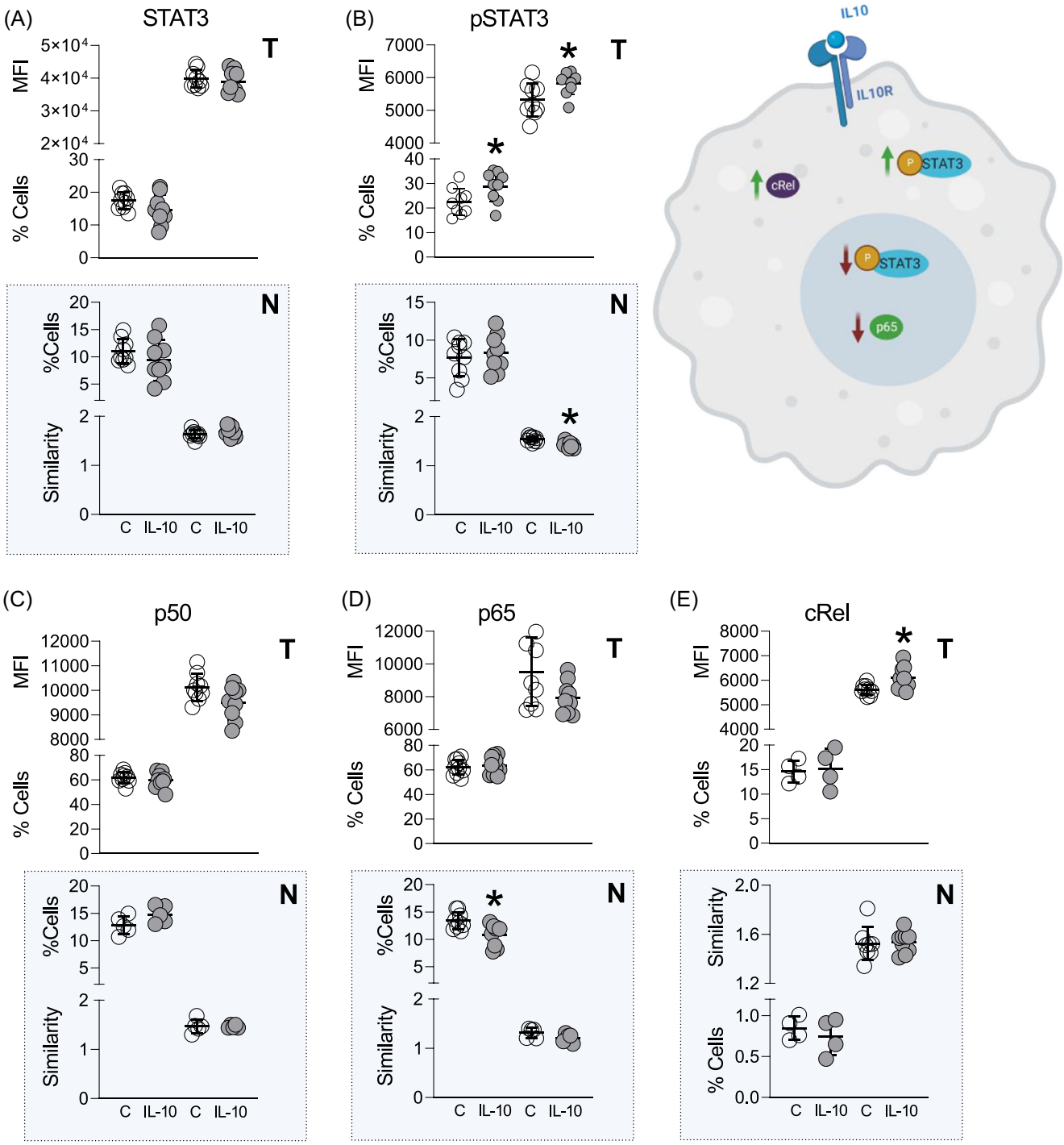

FIGURE 7 IL-10 modulation in peritoneal cells. Total (T) and nuclear (N—blue boxes) expression of STAT3 (A), pSTAT3 (B), p50 (C), p65 (D), and cRel (E) NF-κB subunits of peritoneal cells treated (IL-10) or not (control group, C) with IL-10 3 ng/mL. Data are presented as % of cells expressing the transcriptional factors and as MIF (total cell) or similarity (nucleus) representing expression levels. Results are expressed as mean ± SEM, $n = 8$–10 animals from two independent experiments. Comparisons performed by Student "*t*" test. *$p < .05$ vs. control. The inserted scheme summarizes the main findings (created with BioRender.com).

expressions of pSTAT3 (Figure 7B) and cRel (Figure 7E) and a decrease in the colocalization between STAT3/p50 (Figure 8A), STAT3/p65 (Figure 8B), and pSTAT3/p65 (Figure 8D). A decrease in the nuclear localization of pSTAT3 (Figure 7B) and P65 (Figure 7D), and in the nuclear colocalization of pSTAT3/p65 (Figure 8D) were also observed. IL-10 stimulus did not affect the expressions of STAT3 (Figure 7A) and P50 (Figure 7C).

In spleen cells (Figures 9 and 10), IL-10 reduced pSTAT3 expression (Figure 9B) and STAT3/p50

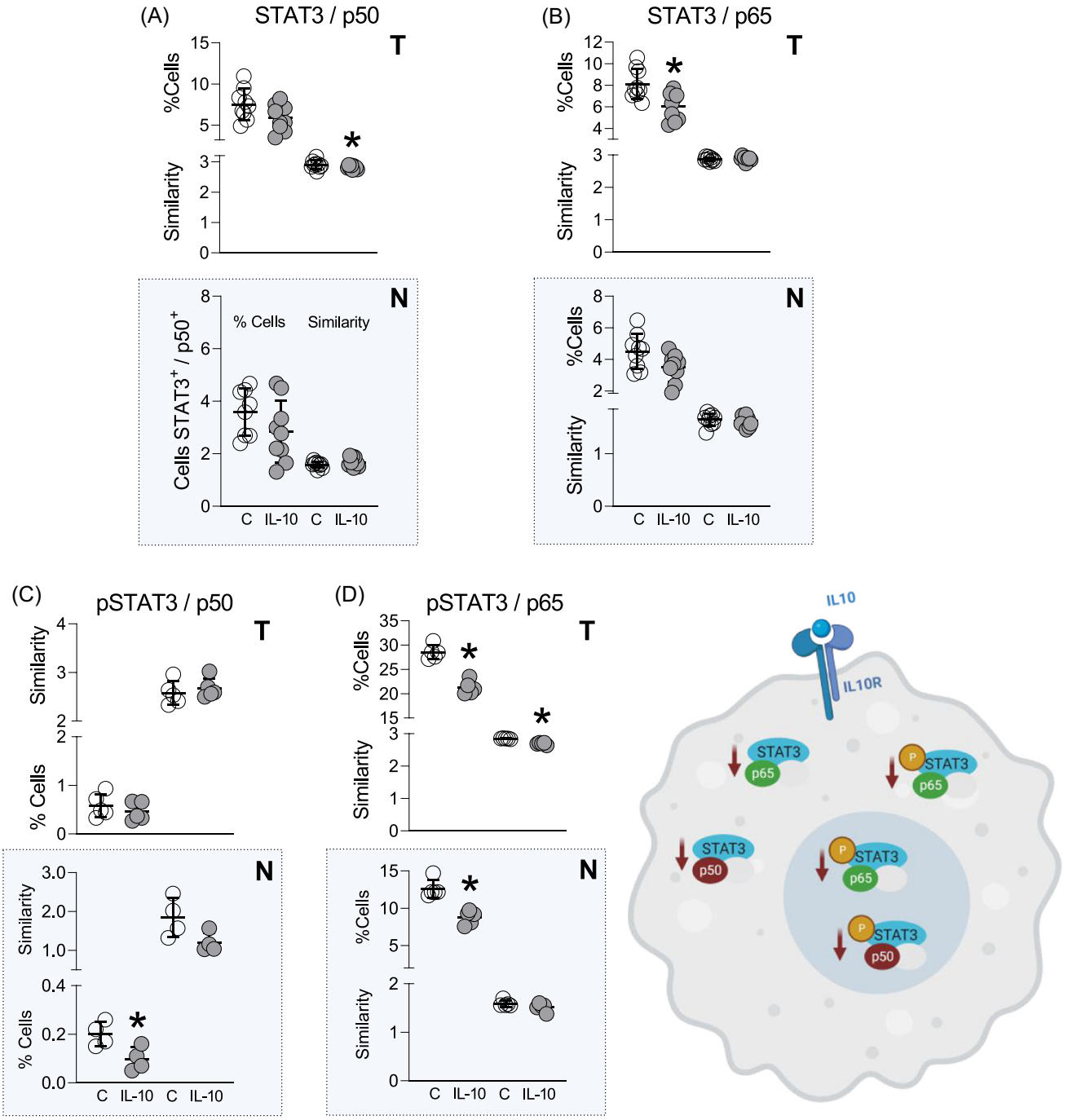

FIGURE 8 IL-10 modulation in peritoneal cells. Total (T) and nuclear (N—blue boxes) colocalizations of STAT3/p50 (A), STAT3/p65 (B), pSTAT3/p50 (C), and pSTAT3/p65 (D) of peritoneal cells treated (IL-10) or not (control group, C) with IL-10 3 ng/mL. Data are presented as % of cells expressing the colocalized transcriptional factors and as similarity scores representing expression levels. Results are expressed as mean ± SEM, $n = 8$–10 animals from two independent experiments. Comparisons performed by Student "*t*" test. *$p < .05$ vs. control. The inserted scheme summarizes the main findings (created with BioRender.com).

colocalization (Figure 10A), increased the cellular colocalization of pSTAT3 with p50 (Figure 10C) and P65 (Figure 10D), the nuclear levels of pSTAT3 (Figure 9B) and P65 (Figure 9D). The expressions of STAT3 (Figure 9A), P50 (Figure 9C), cRel (Figure 9D), and the STAT3/p65 colocalization (Figure 10B) were not altered by IL-10.

Finally, we observed the effect of STAT3 (cucurbitacin I) and NF-κB (PDTC) inhibitors on the expression of the melatonergic enzymes after the IL-10 stimulus. In BM cells, IL-10 treatment does not affect SNAT (Figure 11A) and pSNAT expressions (Figure 11B). However, the increase in the percentage of cells expressing ASMT was reversed by inhibiting STAT3

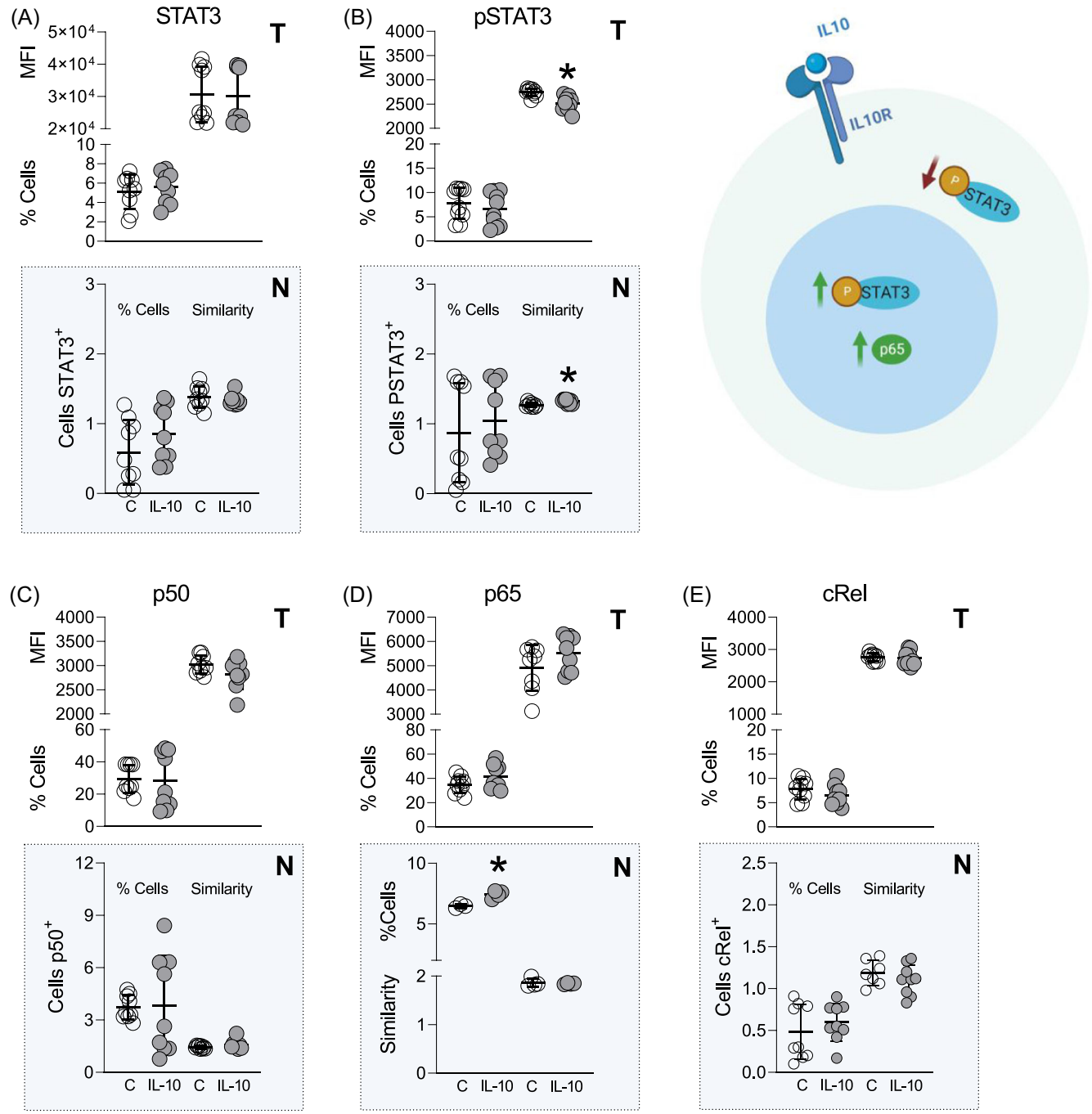

FIGURE 9 IL-10 modulation in spleen cells. Total (T) and nuclear (N—blue boxes) expression of STAT3 (A), pSTAT3 (B) p50 (C), p65 (D), and cRel (E) NF-κB subunits of spleen cells treated (IL-10) or not (control group, C) with IL-10 3 ng/mL. Data are presented as % of cells expressing the transcriptional factors and as MIF (total cell) or similarity (nucleus) representing expression levels. Results are expressed as mean ± SEM, $n = 8$–10 animals from two independent experiments. Comparisons performed by Student "t" test. *$p < .05$ vs. control. The inserted scheme summarizes the main findings (created with BioRender.com).

and NF-κB (Figure 11C). In spleen cells, the SNAT expression was significantly reduced by the inhibition of STAT3 phosphorylation (Figure 11D). The increase in pSNAT expression was reverted by the inhibition of both pathways (Figure 11E). In addition, the negative regulation of ASMT expression was mostly reverted by inhibiting NF-κB activation (Figure 11F). In peritoneal cells, the decrease in the percentage of cells expressing SNAT was partially restored by inhibiting both STAT3 phosphorylation and NF-κB activation (Figure 11G).

The inhibition of STAT3 phosphorylation and NF-κB activation reverted the IL-10-induced increase

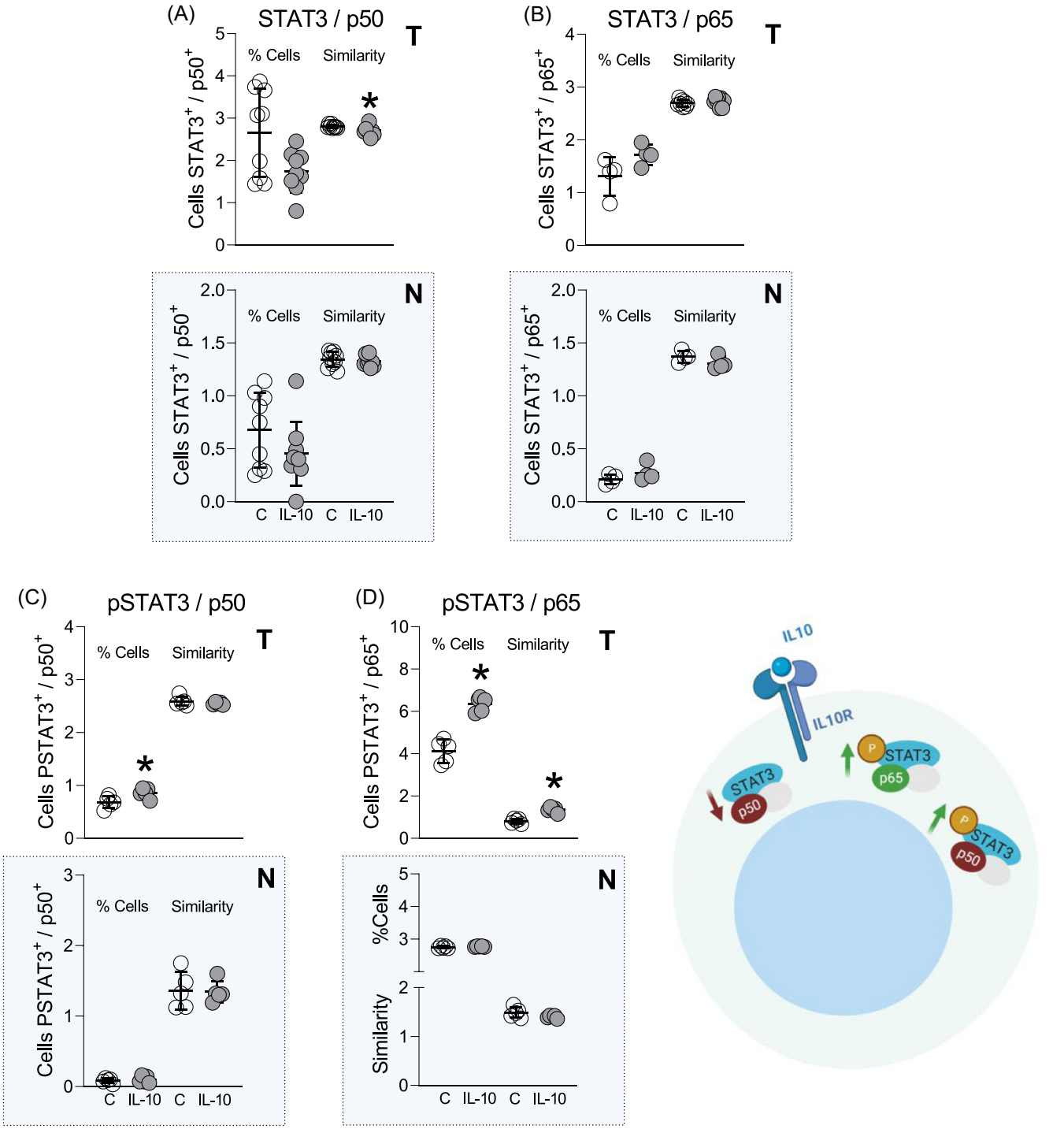

FIGURE 10 IL-10 modulation in spleen cells. Total (T) and nuclear (N—blue boxes) colocalizations of STAT3/p50 (A), STAT3/p65 (B), pSTAT3/p50 (C), and pSTAT3/p65 (D) of spleen cells treated (IL-10) or not (control group, C) with IL-10 3 ng/mL. Data are presented as % of cells expressing the colocalized transcriptional factors and as similarity scores representing expression levels. Results are expressed as mean ± SEM, $n = 8–10$ animals from two independent experiments. Comparisons performed by Student "t" test. *$p < .05$ vs. control. The inserted scheme summarizes the main findings (created with BioRender.com).

in melatonin levels in BM cells (Figure 12A), suggesting that STAT3/NF-κB crosstalk regulates melatonin synthesis in the BM. In the spleen, although the increase in SNAT and pSNAT expression was more related to STAT3 phosphorylation, the increase in melatonin content was reversed only by inhibiting NF-κB activation (Figure 12B). In peritoneal cells, the inhibitors showed that both pathways are involved in the IL-10-induced decrease of melatonin levels (Figure 12C).

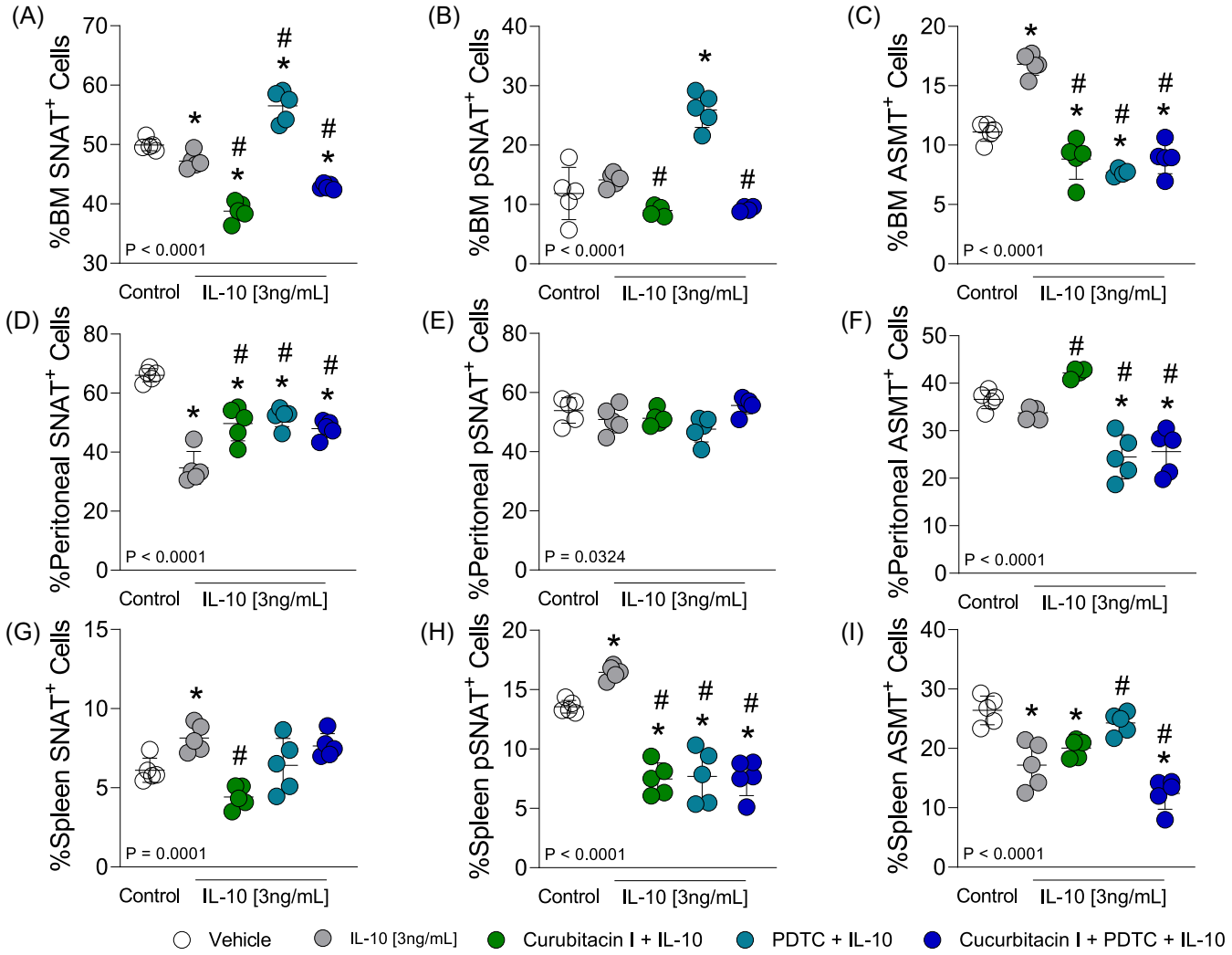

FIGURE 11 IL-10 regulates melatonergic enzymes through STAT3/NF-κB crosstalk in BM (A–C), peritoneal (D–F), and splenic cells (G–I). SNAT, pSNAT, and ASMT expressions were measured after inhibition of STAT3 and/or NF-κB pathway. Cells were pre-incubated or not with a STAT3 phosphorylation inhibitor (Cucurbitacin I, 3 μM) and/or an NF-κB inhibitor (PDTC, 3 μM) for 30 min, then stimulated with IL-10 (3 ng/mL, 3 h). After stimulation, cells were collected for measurement of the enzyme expression by flow cytometry. Results are expressed as mean ± SEM, $n = 8$–10 animals from two independent experiments. Data were analyzed by one-way ANOVA with Tukey's post hoc. $^{*}p < .05$ vs. control; $^{\#}p < .05$ vs. IL-10 (3 ng/mL).

4 | DISCUSSION

The IPA theory has brought to light the relevance of context-dependent regulations of melatonin synthesis.[34] The dynamic complexity of the system allows independent local and systemic modulations of melatonin content and, therefore, efficient coordination of physiologic and pathophysiologic processes. IL-10 and melatonin are pleiotropic molecules that exert context- and cell-dependent stimulatory and/or inhibitory effects upon the immune system.[2,4,35,36] The crosstalk among these signals composes a network of interconnections that coordinate the trimming and intensity of several immunological processes in health, pathophysiological, and pathological conditions. The present work adds relevant information to this scenario, highlighting the cellular mechanisms triggered by IL-10 that adjust melatonin production by immune cells and the pineal gland.

The binding of IL-10 to its receptor (IL-10R) activates the tyrosine kinases Jak1 and Tyk2, which are associated with the IL-10Rα and IL-10Rβ, respectively.[37] Receptor engagement and tyrosine phosphorylation lead to the recruitment and phosphorylation of STAT3, resulting in STAT3 homodimerization and translocation to the nucleus, which acts as a transcription activator.[38] Recently, we showed that the STAT family colocalizes with NF-κB in the *Snat* promoter region, affecting the regulation of NF-κB-induced gene expression and melatonin synthesis.[21] Several studies also reported that STAT3 physically interacts with members of the NF-κB

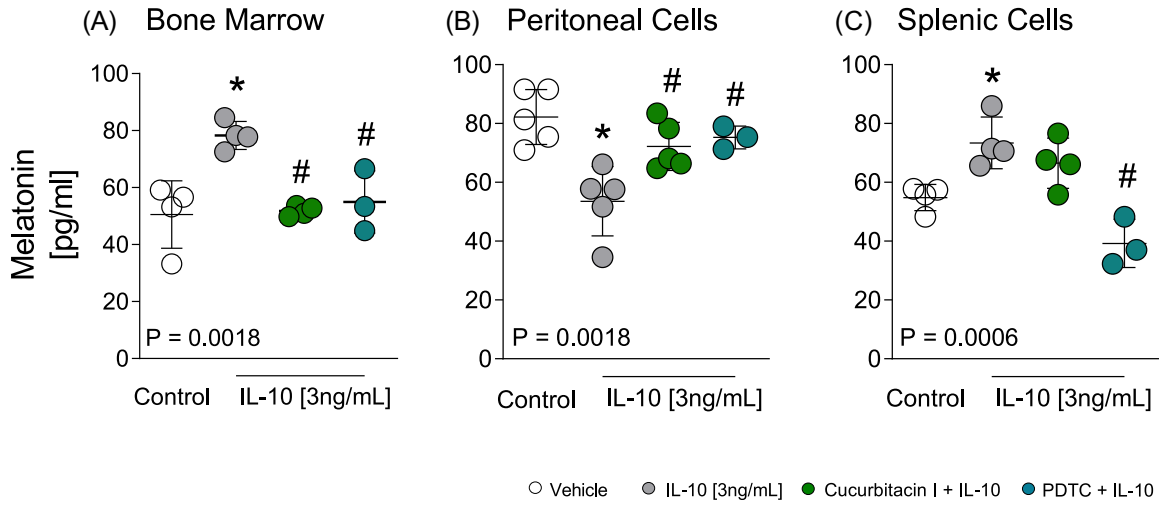

FIGURE 12 IL-10 regulates melatonin levels through STAT3/NF-κB crosstalk in BM (A), peritoneal (B), and splenic cells (C). Melatonin levels were measured after inhibition of STAT3 and/or NF-κB pathway. Cells were pre-incubated or not with a STAT3 phosphorylation inhibitor (Cucurbitacin I, 3 μM) and/or an NF-κB inhibitor (PDTC, 3 μM) for 30 min, then stimulated with IL-10 (3 ng/mL, 5 h). After stimulation, the supernatant was collected for melatonin quantification by ELISA. Results are expressed as mean ± SEM, $n = 3–5$ animals from two independent experiments. Data were analyzed by two-way ANOVA with Tukey's post hoc. $^{*}p < .05$ vs. control; $^{\#}p < .05$ vs. IL-10 (3 ng/mL).

family, notably p65 and p50,[27,39–42] and the results of bioinformatic searches revealed that such an interaction could also occur in the promoter region of Snat and Asmt genes. Interestingly, the effects of IL-10 3 ng/mL on SNAT/pSNAT and ASMT expressions were reversed by inhibiting both STAT3 phosphorylation and NF-κB activation, suggesting that IL-10 is inducing crosstalk between the STAT3 and NF-κB pathways.

Melatonin synthesis regulation by NF-κB-controlled *Snat* transcription has been widely studied on different tissues using different stimuli.[4,19] The nuclear translocation of NF-κB induced by IL-10 (3 ng/mL) was tissue-specific. The p50 and p65 NF-κB translocation was increased in BM, while only a nuclear increase of p65 NF-κB was observed in the spleen. This same subunit was reduced in peritoneal cells. In all cases, the increase in the melatonin synthesis pathway is related to the p65 NF-κB subunit activation, as has been shown previously.[7,18–21,25] Similar results were also observed in studies carried out in the pineal gland,[21] but it is noteworthy that in this and the BM cells, the increase in melatonin synthesis with IL-10 3 ng/mL was directly associated with the increase of ASMT and not (P)SNAT. These results show that NF-κB regulates melatonin synthesis also through the control of ASMT and suggest that a similar mechanism modulates melatonin synthesis in the pineal gland and BM cells.

Regulation of melatonin synthesis occurs depending on the tissue and the cell studied, which may be directly associated with how cells respond to the immune signals. The effect of IL-10 on NF-κB activation has been extensively studied in monocytes/macrophages.[43–46] It is well known that IL-10 inhibits the activation of human and murine macrophages by selectively inducing the p50/p50 NF-κB homodimer translocation, blocking p65 NF-κB subunits nuclear translocation, and inhibiting DNA-binding of the nuclear p65/p50 NF-κB heterodimer.[43,44] These observations are in accordance with the present data regarding peritoneum phagocytes. In contrast, some studies show that IL-10 may also have immune stimulatory effects on B cells, $CD4^+$ and $CD8^+$ T cells, and NK cells,[47–50] and even activate NF-κB in $CD8^+$ T cells.[51] Therefore, it could be hypothesized that the differences found within the locals evaluated in the present study are due to the effect that IL-10 has on the activation of p50 and p65 NF-κB subunits in different types of cells that produce melatonin locally.

The modulation of IL-10 on the melatonergic system in lymphoid tissues would be more related to immunological adjustments in the earliest and late phases of the process. In the BM, melatonin locally produced adjusts hematopoietic stem cell proliferation, differentiation in lymphoid and myeloid progenitors, and cellular migration.[52] In the spleen, although there is limited information on the role of local production,[26] studies performed with exogenous melatonin show increased splenic production of IL-10[53] and splenocyte proliferation,[53] and lower sepsis-induced apoptosis.[54] Melatonin also inhibits Th1 and Th17 responses and increases regulatory

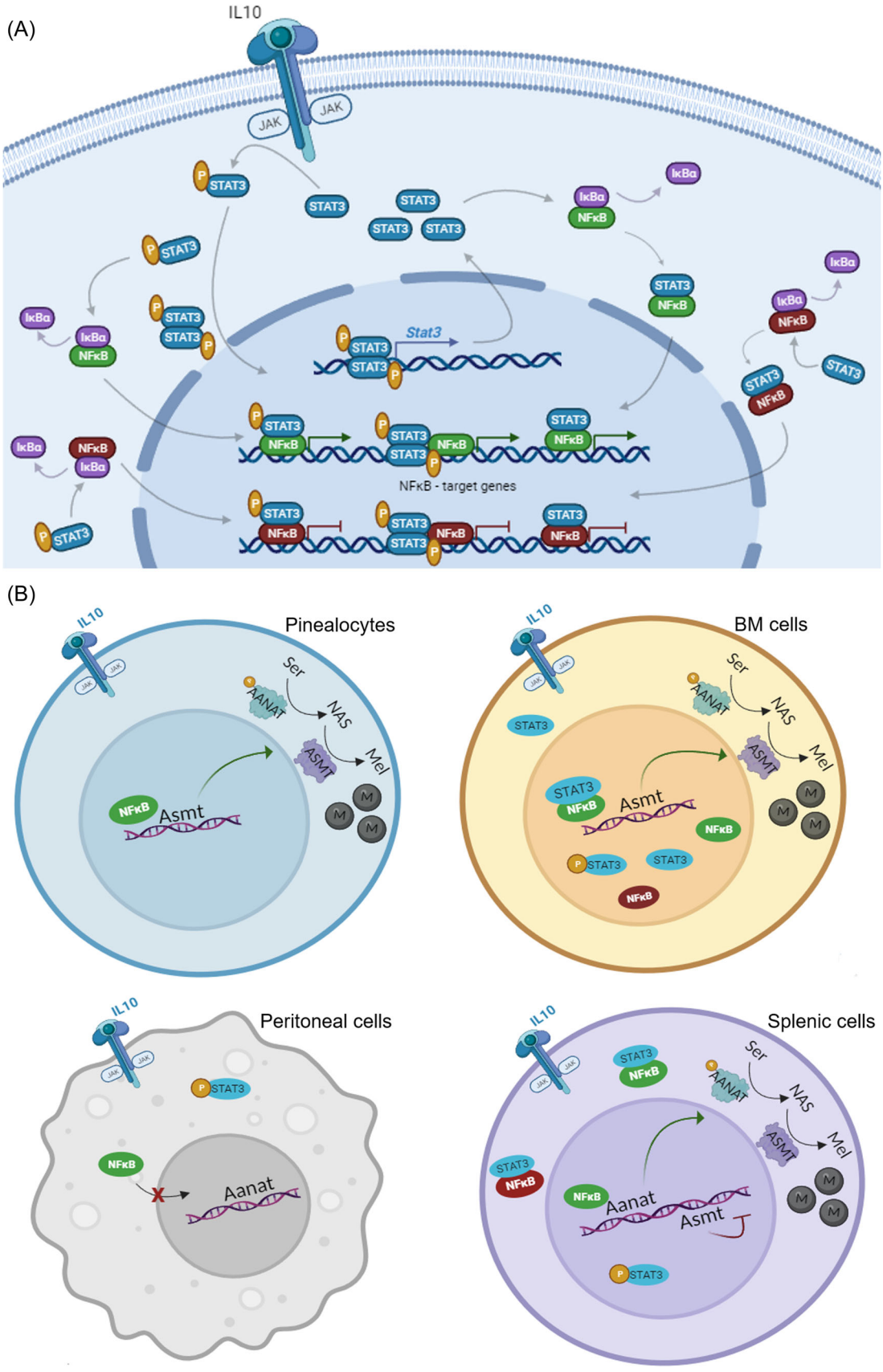

FIGURE 13 (See caption on next page).

cells in some inflammatory diseases,[55] through a mechanism associated with increased differentiation of IL-10-producing type 1 Treg cells.[56] In peripheral phagocytes, a mutual IL-10/melatonin modulation could be present. TNF-induced TAD^+ NF-κB activation increases melatonin synthesis and phagocytosis.[18–20] Melatonin positively regulates the expression of IL-10 in several models of inflammation,[57–60] reducing TNF levels[60] and favoring the polarization of macrophages to the M2 recovery profile. Therefore, the inhibitory actions of IL-10 upon melatonin adjust melatonin production during the assembly phase and participate in the restoration to the initial conditions during the recovery phase of the inflammatory process.

The IPA theory considers the bidirectional modulation of melatonergic and immune systems during different phases of the immune response. At the onset of inflammation, elevated levels of TNF,[61] together with the combination of α_1-adrenergic stimulation and high GC levels,[61] favor the inhibition of pineal melatonin synthesis, allowing the migration of immune cells to the sites where they are required. In this context, regulatory actions of IL-10 would be already present but when inhibitory signals are removed (latter decrease of TNF and α-adrenergic stimulation), the pathways triggered by IL-10 (present data), IFN-γ,[21] and GC[12,22,62] could synergize to restore pineal melatonin synthesis. Therefore, this return will depend on a combination of different immunological signals along with correct adrenergic stimulation.

In unbalanced immune responses, the melatonergic system and the rhythm produced by the pineal could be altered by the overproduction of IL-10 along with altered levels of other immune signals, such as TNF and GC. In this context, our results indicate that IL-10 (100 ng/mL) could have an effect almost opposite to that observed at lower levels (3 ng/mL), decreasing melatonin produced locally by the BM, spleen, and peritoneal cells without affecting pineal hormonal synthesis. A possible mechanism that could help explain this result would be that at higher concentrations, the binding affinity of IL-10 for IL-10Rβ increases, favoring receptor heterodimerization and a more potent activation of STAT3.[63] Thus, the increase in the STAT3 pathway could lead to more p50/p50 NF-κB homodimers being activated and translocated to the nucleus,[43] reducing the melatonin synthesis pathway. Further investigation focusing on the sensitivity of different immune cells to IL-10 will add valuable information regarding melatonergic modulation in health and disease conditions.

In septic shock, the rhythmic production of melatonin is reduced.[64,65] The higher production of IL-10 is associated with poor outcomes in septic patients as this cytokine induces later immune suppression, rendering the subjects more susceptible to infections.[66–68] In this context, an inhibitory effect of IL-10 on the production of melatonin by the pineal gland and immune cells might be favoring the dysregulation of the immune response. The development of several types of cancer is also associated with impaired activation of the immune system characterized by a lack of antigen-specific immunity and intratumoral $CD8^+$ T cells, but also with a high prevalence of infiltrating macrophages and chronic inflammation mediators in late-stage patients.[69,70] The pleiotropic actions of IL-10 in tumor progression are widely investigated and anti-IL-10 therapies presented beneficial effects.[71–73] Tumor progression can be also affected, among other things, by the reduction in nocturnal melatonin levels that impair the circadian rhythm.[74–77] Our findings then suggest that a reduction in IL-10 levels could induce an increase in pineal melatonin and its rhythm amplitude, as well as melatonin produced locally by non-phagocytic cells, which in solid tumors is associated with a better prognosis.[78,79] High levels of IL-10 are also observed and correlated with a worse prognosis in later phases in burn patients,[80] neurodegenerative diseases[81] such as Parkinson's disease,[82] ischemic stroke,[83] Huntington's disease,[84] and autoimmune diseases like lupus erythematosus[85] and rheumatoid arthritis.[86] Interestingly, the circadian rhythm of melatonin is also impaired in these pathological conditions. Therefore, the evaluation of how IL-10/melatonergic system interactions modulate the progression of chronic inflammatory disease could provide some interesting insights for new therapeutic strategies.

In conclusion, the present findings are the first to propose a mechanism by which lower levels of IL-10 regulate melatonin synthesis through the (P)STAT3/NF-κB crosstalk (Figure 13) and to propose that, in the course of

FIGURE 13 IL-10/STAT3/NF-κB interactions. (A) IL-10 binding recruits STAT3, which is additionally phosphorylated by JAK1. pSTAT3 homodimerizes, moves to the cellular nucleus and activates gene transcription, including the STAT3 gene itself. The increase of STAT3 and pSTAT3 cytoplasmic can lead to the interaction with inactive NF-κB, resulting in NF-κB activation and translocation into the nucleus (green: NF-κB TAD^+, red: NF-κB TAD^-). On the other hand, pSTAT3 homodimers already translocated to the nucleus can colocalize with NF-κB in the NF-κB-regulated genes promoter region. In either case, (p)STAT3 or pSTAT3 homodimers' interaction with NF-κB in the promoter region induces an increase in the time in which NF-κB binds to DNA, leading to an increase in the expression of NF-κB-target genes for NF-κB TAD^+ dimers (green) or an increased inhibition of NF-κB-target gene expression for NF-κB TAD^- dimers (red). (B) Effects of IL-10 on melatonin synthesis on pinealocytes, BM, peritoneal and splenic cells. Created with BioRender.com.

an immune response, IL-10 adjusts melatonin synthesis depending on the local context. The bidirectional communication between IL-10 and melatonin in peripheral and lymphoid tissues seems to regulate the acute and resolution phases of inflammatory processes, which, together with the regulation exerted by GCs in the pineal, may be key to a better and broader understanding of the pineal immune axis during the immune response. In this sense, it would be important to evaluate whether an unbalance of IL-10/melatonin/GC interaction could be associated with tumor development, lethal acute or chronic inflammatory processes, as the overproduction of IL-10 and GC in septic models is associated with increased immunosuppression[87] and the impaired GC/melatonin crosstalk render animals more susceptible to rheumatoid arthritis development.[88]

AUTHOR CONTRIBUTIONS

Marlina O. Córdoba-Moreno, Regina P. Markus, and Pedro Augusto C. M. Fernandes conceived and designed the experiments. Marlina O. Córdoba-Moreno, Gabriela Christine Santos, and Débora dos Santos-Silva performed the experiments. Marlina O. Córdoba-Moreno, Sandra M. Muxel, Caroline L. Quiles, Kassiano D. S. Sousa, Regina P. Markus, and Pedro Augusto C. M. Fernandes analyzed data and statistics. Marlina O. Córdoba-Moreno, Regina P. Markus, and Pedro Augusto C. M. Fernandes wrote the manuscript. All authors critically revised the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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