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Serotonin (5-HT) shapes the macrophage gene profile through the 5-HT_{2B}-dependent activation of the Aryl Hydrocarbon Receptor (AhR)

Short title: 5-HT_{2B} shapes the macrophage transcriptome via AhR

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ABSTRACT

Macrophages can either promote or resolve inflammatory responses, and their polarization state is modulated by peripheral serotonin (5-hydroxytryptamine, 5-HT). In fact, pro- and anti-inflammatory macrophages differ in the expression of serotonin receptors, with 5-HT_{2B} and 5-HT_7 expression restricted to IL-10-producing human macrophages (M-MØ). 5-HT_7 drives the acquisition of pro-fibrotic and anti-inflammatory functions in M-MØ, whereas 5-HT_{2B} prevents the degeneration of spinal cord mononuclear phagocytes and modulates motility of murine microglial processes. Since 5-HT_{2B} mediates clinically relevant 5-HT-related pathologies (valvular heart disease, pulmonary arterial hypertension) and is an off-target of anesthetics, anti-parkinsonian drugs and Selective Serotonin Reuptake Inhibitors (SSRI), we sought to determine the transcriptional consequences of 5-HT_{2B} engagement in human macrophages, where 5-HT_{2B} signaling remains unknown. Assessment of the effects of specific agonists and antagonist revealed that 5-HT_{2B} engagement modifies the cytokine and gene signature of anti-inflammatory M-MØ, upregulates the expression of Aryl hydrocarbon Receptor (AhR) target genes, and stimulates the transcriptional activation of AhR. Moreover, we found that 5-HT dose-dependently upregulates the expression of AhR target genes in M-MØ, and that the 5-HT-mediated activation of AhR is 5-HT_{2B}-dependent because it is abrogated by the 5-HT_{2B}-specific antagonist SB204741. Altogether, our results demonstrate the existence of a functional 5-HT / 5-HT_{2B} / AhR axis in human macrophages and indicate that 5-HT potentiates the activity of a transcription factor (AhR) that regulates immune responses and the biological responses to xenobiotics.

INTRODUCTION

Peripheral serotonin (5-hydroxytryptamine, 5-HT) influences the physiopathology of numerous tissues [1-3] as it controls vascular, heart and gastrointestinal functions [4,5], promotes cell proliferation [6-9], regulates wound healing, and influences immune and inflammatory responses [10] by modulating T lymphocyte [11] and myeloid cell functions [12-16]. In inflammatory pathologies, 5-HT contributes to Pulmonary Arterial Hypertension (PAH) [17], atopic dermatitis [18], systemic sclerosis [19], inflammatory gut disorders [20-25], cancer angiogenesis [26] and neuroendocrine neoplasms proliferation [27], whereas it reduces pathologic scores in collagen-induced arthritis [28]. The existence of seven types of 5-HT receptors (5-HT_{1-7}), with different distribution and signaling properties [29], underlies these tissue-specific actions of 5-HT.

Macrophages promote the initiation and the resolution of inflammatory processes, with both processes being essential for maintaining tissue homeostasis [30-32]. In fact, modulation of macrophage polarization is an attractive therapeutic target for chronic inflammatory pathologies, where the balance between pro-inflammatory and resolving macrophages is altered [33,34]. Not surprisingly, macrophage effector functions are directly modulated by 5-HT [17,22,26,35,36], and also by Selective Serotonin Reuptake Inhibitors (SSRI) [37,38]. We have previously demonstrated that 5-HT_7 is the major mediator of the anti-inflammatory actions of 5-HT on human macrophages [39] and that both 5-HT_{2B} and 5-HT_7 contribute to the maintenance of the anti-inflammatory state of M-CSF-dependent human macrophages [40].

The 5-HT_{2B} serotonin receptor is expressed in the central nervous system, where it exerts anti-depressant or anxiolytic-like effects [41], and in periphery, where it plays a major role in heart development [42]. Pharmacologic and genetic studies indicate that 5-HT_{2B} is required for 5-HT-mediated pathologies like PAH [17,43], dermal, lung and liver fibrosis [19,44,45], cardiac hypertrophy

[46,47], and mediates the valvular heart disease (VHD) and fibrosis associated with carcinoid syndrome [48]. Importantly, anti-migraine drugs like methysergide and ergotamine [49,50], general anesthetics [51], fenfluramine and conventional SSRIs [52-55], and even the dopamine agonists and anti-parkinsonian drugs pergolide and cabergoline [50,56,57], exert potent off-target effects on 5-HT_{2B} [50]. In fact, 5-HT_{2B} appears to be required for the therapeutic actions of SSRI [54]. Since some of these 5-HT_{2B}-targeting drugs induce VHD, their use is restricted, and novel potential drugs are commonly screened for 5-HT_{2B} agonist activity.

5-HT_{2B} engagement enhances proliferation of numerous cell types [9,58-67] via Gαq and Src phosphorylation, and through production of growth factors like insulin [68], TGFβ1, CTGF, FGF2 [45,60] and TGFα [69]. In macrophages, 5-HT_{2B} prevents mononuclear phagocyte degeneration in amyotrophic lateral sclerosis [70], and *Htr2b*$^{-/-}$ microglia exhibits a mild inflammatory state [71], which is in line with the ability of 5-HT_{2B} antagonist SB204741 to impair the acquisition of human macrophage polarization-specific genes [40] and TGFβ1 expression by mouse Kupffer cells [45]. However, although 5-HT_{2B} activation modifies inflammatory cytokine production by human monocytes [72], the functional and transcriptional consequences of 5-HT_{2B} ligation in human macrophage remains to be determined. Since anti-inflammatory M-CSF-dependent macrophages express 5-HT_{2B}, we have assessed the specific role of 5-HT_{2B} in macrophage polarization, and found that both serotonin and the 5-HT_{2B} agonist BW723C86, used to evaluate the role of 5-HT_{2B} in preclinical models of depression [54] and anxiety [73,74], are capable of shaping the macrophage transcriptome via Aryl hydrocarbon Receptor (AhR) activation.

EXPERIMENTAL PROCEDURES

Generation of human monocyte-derived macrophages and cell culture. Buffy coats from anonymous healthy blood donors were provided by Comunidad de Madrid blood Bank. Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats over a Lymphoprep (Nycomed Pharma) gradient, and monocytes were purified by magnetic cell sorting using CD14 microbeads (Miltenyi Biotech). Monocytes (>95% $CD14^+$ cells) were cultured at 0.5 x 10^6 cells/ml for 7 days in RPMI supplemented with 10% fetal calf serum (FCS, Biowest) (completed medium) at 37ºC in a humidified atmosphere with 5% CO_2, and containing M-CSF (10 ng/ml) (ImmunoTools GmbH) to generate M-MØ monocyte-derived macrophages. M-CSF was added every two days. Before treatments, M-MØ were maintained in serum-free medium (Macrophage-SFM, Gibco) for 48 hours. For macrophage activation, cells were exposed to 5-HT_{2B} agonists for 6 hours and then treated with *Escherichia coli* 055:B5 LPS (10 ng/ml) for 18 h. The 5-HT_{2B} agonists BW723C86 (α-methyl-5-(2-thienylmethoxy)-1*H*-indole-3-ethanamine) [75,76] and α-Methylserotonin (AMS) [77], and the 5-HT_{2B} antagonist SB204741 [78], were purchased from Sigma-Aldrich. The 5-HT_{2B} agonist 6-APB (6-(2-aminopropyl)benzofuran) was obtained from Cayman and used at the indicated concentrations. The AhR agonist FICZ (Enzo) and antagonist CH223191 (Calbiochem) were used at 250 nM and 3 μM, respectively. When indicated, SB204741 was used 1 hour before treatment with agonists. Monocyte-derived osteoclasts were generated by culturing monocytes for 12 days on glass coverslips with M-CSF (25 ng/ml) and Receptor Activator for NF-kB Ligand (RANKL; 30 ng/ml) addition every 72 h and BW723C86 treatment 6 h before cytokine addition. Osteoclast generation was verified directly by phase contrast microscopy or after staining for Tartrate-Resistant Acid Phosphatase (TRAP) (Leukocyte Acid Phosphatase kit, Sigma-Aldrich). Human TNFα and CCL2 in M-MØ culture supernatants were measured using commercially available ELISA (BD Biosciences).

Transfections and Reporter gene assays. The Cignal® Xenobiotic Response Element (XRE), where the XRE-Luc construct harbours tandem repeats of the AhR-binding element (XRE), was used for the reporter gene assays. HepG2 cells (80.000 cells/well) or M-MØ ($1x10^6$ cells/well) were transfected with 1 μg of the XRE reporter construct using either Superfect (Qiagen) or VIROMER RED (Lipocalix), respectively. For normalizing transfection efficiency, transfected DNA included a 40:1 mixture of the XRE-specific firefly luciferase construct and a construct expressing *Renilla* luciferase from a constitutive promoter. After transfection, cells were cultured overnight before exposure to BW723C86 (10 μM), FICZ (250 nM), CH223191 (3 μM) or DMSO for 24 h, lysed, and firefly and *Renilla* luciferase activities were determined using the Dual-Luciferase® Reporter Assay System (Promega).

Small Interfering Ribonucleic Acid (siRNA) Transfection. To knockdown AhR expression, M-MØ (1×10^6 cells) were transfected with AhR-specific siRNA (siAhR) (50 nM) (# s1199, Thermo-Fisher Scientific), using HiPerFect (Qiagen) using siC (#4390843, Thermo-Fisher Scientific) as negative control siRNA. Cells were allowed to recover from transfection in complete medium (18–24 h) and then treated for 6h with BW723C86 (10 μM) before analysis.

Real-time quantitative RT-PCR (qRT-PCR). Total RNA was extracted using the total RNA and protein isolation kit (Macherey-Nagel). RNA samples were retrotranscribed (High-Capacity cDNA Reverse Transcription kit, AB), and triplicates of amplified cDNA were analyzed on a Light Cycler® 480. Gene-specific oligonucleotides (Table S1) were designed using the Universal ProbeLibrary software (Roche Diagnostics). Results were expressed relative to the expression level of the endogenous reference gene *TBP* and using the ΔΔCT (cycle threshold) method for quantitation.

Microarray and RNAseq analysis. Global gene expression analysis was performed on RNA from three independent samples of untreated (control), BW723C86-treated (10 μM), SB204741-treated (1 μM), or SB204741+BW723C86-treated M-MØ. RNA was isolated using the RNeasy Mini kit (Qiagen) and used as hybridization probe on Whole Human Genome Microarrays (Agilent Technologies, Palo Alto, CA). Only probes with signal values >60% quantile in at least one condition were considered for the differential gene expression (DGE) and statistical analysis. Statistical analysis for DGE was carried out using empirical Bayes moderated paired t-test implemented in the *limma* package (http://www.bioconductor.org), and p values were adjusted for multiple hypotheses testing using the Benjamini-Hochberg method to control the false discovery rate [79], with all procedures coded in R (http://www.r-project.org). Microarray data were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE121825. RNAseq was performed on three independent samples of M-MØ exposed for 6 hours to 10 μM BW723C86, 10 μM 5-HT or 100 μM 5-HT and processed at BGI (https://www.bgitechsolutions.com) using the Illumina HiSeq platform. An average of 5.41Gb were generated per sample and, after filtering, clean reads were mapped to the reference (UCSC Genome assembly hg38) using HISAT2/ Bowtie2 (average mapping ratio 93.41%) [80,81]. Differential gene expression was assessed by using DEseq2 algorithms using the parameters Fold Change>2 and adjusted p value <0.05. Heatmaps and clustering were done using the Genesis software (http://genome.tugraz.at/genesisclient/) [82]. Differentially expressed genes were analysed for annotated gene sets enrichment using ENRICHR (http://amp.pharm.mssm.edu/Enrichr/) [83,84], and enrichment terms considered significant with a Benjamini-Hochberg-adjusted *p* value <0.05. For gene set enrichment analysis (GSEA) [85], gene sets available at the website, as well as gene sets generated from publicly available transcriptional studies (https://www.ncbi.nlm.nih.gov/gds), were used.

Statistical analysis. For comparison of means, and unless otherwise indicated, statistical significance of the generated data was evaluated using the Student paired t-test. In all cases, $p<0.05$ was considered as statistically significant.

RESULTS

The BW723C86 agonist modulates the transcriptional signature of human macrophages partially via 5-HT_{2B}. Human anti-inflammatory M-CSF-primed macrophages (M-MØ) express 5-HT_7 and 5-HT_{2B} serotonin receptors [40], and both contribute to the acquisition of the M-MØ-specific transcriptome [39,40]. Since 5-HT_{2B} is the target of clinically relevant drugs (anesthetics, SSRIs, antimigraine drugs) [49,51-54], we sought to determine the consequences of 5-HT_{2B} engagement in human macrophages. To that end we made use of BW723C86, an 5-HT_{2B} agonist with 10-fold and 100-fold selectivity over human 5-HT_{2C} and 5-HT_{2A}, respectively [86], amply used as *in vivo* [54,73,74,87-92] and *ex vivo* [28,66,70], and with potential therapeutic effects [73,74,92]. Transcriptional profiling of BW723C86-treated M-MØ (Figure 1A) revealed that the agonist increased the expression of 357 annotated genes and downregulated the expression of 401 genes (*adj* $p<0.002$) (Figure 1B, Tables S2 and S3). Gene Set Enrichment Analysis (GSEA) revealed that BW723C86 significantly modifies the expression of "$G\alpha_{12/13}$ signaling" [93] and "Amyotrophic Lateral Sclerosis" gene sets, in agreement with the signaling capability and pathological significance of 5-HT_{2B} [70] (Figure S1), and affects the expression of genes associated to the terms "GO Heart Valve Development" and "GO Regulation of Myeloid Leukocyte Differentiation" (Figure S1), in line with the involvement of 5-HT_{2B} in heart development [42,94,95] and macrophage differentiation [40].

To identify the genes whose expression is regulated by BW723C86 in a 5-HT_{2B}-dependent manner, the transcriptional effects of the 5-HT_{2B} agonist were also determined in the presence of SB204741, a selective 5-HT_{2B} antagonist with 100-fold selectivity over 5-HT_{2C} and 5-HT_{2A} [86] that is widely used *in vivo* [9,61,63,91,96] (BW723C86 + SB204741; Figure 1A, Table S4). We reasoned that the genes significantly modulated by BW723C86, but not by BW723C86+SB204741, would represent *bona fide* 5-HT_{2B}-regulated genes. In the presence of

the antagonist SB204741, BW723C86 significantly (*adj p<0.002*) increased the expression of 155 annotated genes and downregulated the expression of 70 genes (Figure 1B, Table S5). Comparison of the BW723C86-induced transcriptional changes in the absence or presence of the antagonist (Figure 1C,D) indicated that the antagonist SB204741 prevents the BW723C86-mediated upregulation of 217 genes, and the BW723C86-mediated downregulation of 334 genes (Figure 1C,D, Table S6). Therefore, 5-HT_{2B} mediates the enhanced expression of 60% of the genes upregulated by BW723C86 (217 genes) and the diminished expression of 84% of the genes downregulated by BW723C86 (Figure 1E, Table S6). These results also point towards 5-HT_{2B}-independent effects of BW723C86, as it modulates the expression of 207 genes (140 upregulated, 67 downregulated) even in the presence of the 5-HT_{2B} antagonist (Figure 1C,D, Table S6). Altogether, these results demonstrate that BW723C86 modifies the macrophage transcriptome through both 5-HT_{2B}-dependent (SB204741-sensitive) and 5-HT_{2B}-independent mechanisms.

BW723C86 modifies the LPS-induced cytokine and gene profile. As GSEA revealed that BW723C86 downregulates the "TNFα Signaling via NFκβ" and "Inflammatory Response" gene sets (Figure S1), we next assessed the ability of BW723C86 to alter macrophage responses to an inflammatory stimulus like LPS (Figure S2A). Although BW723C86 did not modify basal cytokine production by M-MØ (Figure S2B and not shown), it significantly reduced pro-inflammatory TNFα production, and increased anti-inflammatory CCL2 release [97], from LPS-treated M-MØ (Figure S2B). A similar trend was observed with α-Methyl Serotonin (AMS), a less selective 5-HT_{2B} agonist [77], although its effects did not reach statistical significance (data not shown). Importantly, the effect of BW723C86 on cytokine production by LPS-activated M-MØ was blunted by the 5-HT_{2B} antagonist SB204741 (Figure S2B), thus confirming that BW723C86 modifies the LPS-induced cytokine profile via 5-HT_{2B} engagement. In fact,

BW723C86 significantly impaired the acquisition of the characteristic transcriptome of LPS-stimulated M-MØ (GSE99056) [98], an effect that was also abolished by the 5-HT_{2B} antagonist SB204741 (Figure S2C). Thus, engagement of 5-HT_{2B} by BW723C86 conditions human macrophages for altered responses to LPS.

BW723C86 upregulates the expression of AhR target genes via 5-HT_{2B}. Further analysis of the transcriptome of BW723C86-treated M-MØ by GSEA and Enrichr (http://amp.pharm.mssm.edu/Enrichr/) [83,84] revealed a positive enrichment of genes associated with the terms "Metabolism of xenobiotics by Cytochrome P450" gene set (Figure S1), "Aryl Hydrocarbon Receptor Pathway_Homo sapiens_WP2873" and "Aryl Hydrocarbon Receptor_Homo sapiens_WP2586" gene sets (not shown), suggesting that BW723C86 influences the expression of genes regulated by the Aryl Hydrocarbon receptor (AhR) [99], the major regulator of the biological responses to xenobiotics [100]. Indeed, the 217 genes upregulated by BW723C86 in a SB204741-sensitive manner included AhR target genes like *CYP1B1* and *AHRR* (Figure 1E), and GSEA evidenced that exposure of M-MØ to the 5-HT_{2B} agonist BW723C86 leads to enhanced expression of genes whose expression is upregulated by the AhR agonists Benzopyrene (in human macrophages) and VAF-347 (in human dendritic cells) [99,101] (Figure 2A, upper panels), and that this positive enrichment was prevented in the presence of the 5-HT_{2B} antagonist SB204741 (Figure 2A, lower panels). In line with GSEA results, the BW723C86-mediated upregulation of paradigmatic AhR target genes like *CYP1B1*, *AHRR* and *SEMA6B* [102] was prevented in the transcriptome of BW723C86-treated M-MØ that had been previously exposed to the 5-HT_{2B} antagonist SB204741 (Figure 2B, Tables S2 and S3).

Based on the above findings, we assessed the ability of BW723C86 to upregulate the expression of xenobiotic response genes in independent validation samples, and found that exposure to

BW723C86 upregulates AhR target genes (*AHRR*, *CYP1B1*, *SERPINB2*, *TIPARP*, *EREG*) [99,102] in M-MØ (Figure 2C). The upregulation of AhR-target genes was detected as early as 2 hours after addition of BW723C86 (Figure 2D). More importantly, and in agreement with microarray data, the upregulation of AhR-target genes by BW723C86 was significantly impaired by the 5-HT_{2B} antagonist SB204741 (Figure 2E). Altogether, these results demonstrated that the BW723C86 agonist upregulates the expression of AhR target genes in human macrophages in a 5-HT_{2B}-dependent manner.

The 5-HT_{2B} agonist BW723C86 enhances the transcriptional activity of AhR in M-MØ. Dose-response analysis of the responsiveness of M-MØ to BW723C86 revealed that the 5-HT_{2B} agonist promotes the upregulation of AhR-target genes at concentrations ranging between 0.1 μM and 10 μM (Figure 3A). Thus, we next evaluated whether exposure to BW723C86 leads to AhR activation in M-MØ. To that end, the transcriptional effects of BW723C86 were determined after either pharmacological or siRNA-mediated inhibition of AhR in M-MØ. Pre-treatment with the AhR antagonist CH223191 [103] prevented the BW273C86-induced upregulation of both *CYP1B1* and *TIPARP*, whose expression could be also increased by the AhR agonist FICZ [104] (Figure 3B). Besides, siRNA-mediated knockdown of AhR (Figure 3C,D) completely prevented the BW273C86-induced upregulation of the AhR-targets genes *CYP1B1* and *TIPARP* (Figure 3E). Further supporting the involvement of AhR in the BW723C86-induced upregulation of AhR-target genes, reporter gene experiments revealed that BW723C86 specifically enhances the transcriptional activity of AhR in M-MØ, an effect that was abrogated by the AhR antagonist CH223191 (Figure 3F).

To find out whether the ability of the 5-HT_{2B} serotonin receptor agonist BW723C86 to activate AhR is cell type-specific, a similar set of experiments was performed on 5-HT_{2B}-expressing

human hepatoma HepG2 cells. As shown in Figure 4A, BW723C86 enhanced the expression of *CYP1B1* in HepG2 cells after a 4-6 hr exposure. The 5-HT_{2B} agonist significantly potentiated the transcriptional activity of AhR in HepG2 cells, and the BW723C86-induced AhR transcriptional activity was prevented in the presence of the 5-HT_{2B} antagonist SB204741 (Figure 4B). Moreover, the BW723C86-induced AhR transcriptional activity in HepG2 was greatly impaired by the AhR antagonist CH223191 (Figure 4C). Therefore, engagement of the 5-HT_{2B} serotonin receptor by BW723C86 results in enhancement of the transcriptional activity of AhR and upregulate the expression of AhR-target genes.

Additional 5-HT_{2B} ligands influence the expression of BW723C86-regulated genes. To further support the involvement of 5-HT_{2B} in the ability of BW723C86 to promote AhR activation, the influence of other 5-HT_{2B} agonists on the expression of AhR target genes was determined. Albeit to a lower extent than BW723C86, the potent 5-HT_{2B} agonist 6-APB (10nM-1μM) upregulated *CYP1B1* expression in M-MØ (Figure 5A). Similarly, the 5-HT_{2B} agonist alpha-Methyl-Serotonin (AMS) enhanced macrophage *CYP1B1* expression in a dose-dependent manner, and its effect was abolished by the SB204741 antagonist (Figure 5B). Next, we also tested whether 5-HT had an impact on the BW723C86-induced transcriptional effects, and found that treatment with 10 μM 5-HT but did not modify the expression of *CYP1B1* (Figure 5C). However, the BW723C86-mediated upregulation of *CYP1B1* was reduced by 100 μM 5-HT, whose presence caused a slight increase in the expression of the AhR target gene *CYP1B1* (Figure 5C). These results indicated that 5-HT_{2B} agonists are capable of upregulating AhR target gene expression in a 5-HT_{2B}-dependent manner in human macrophages, and suggested that the presence of 5-HT interferes with the ability of the 5-HT_{2B} agonist BW723C86 to upregulate AhR target genes in human macrophages.

Serotonin increases the expression of AhR target genes in a dose-dependent manner and through interaction with 5-HT_{2B}. Given the above results, and to address whether 5-HT influences the expression of AhR target genes, we initially determined the transcriptome of M-MØ exposed to BW723C86, 10μM 5-HT or 100 μM 5-HT using RNAseq. The expression of the AhR target genes was variably elevated in response to each stimulus, but a core of AhR-regulated genes (including *SERPINB2, CYP1B1, GPR183, SEMA6B* and *IL1B*) was enhanced by all three treatments (Figure 6A). In fact, the transcriptome of BW723C86- and 5-HT-treated M-MØ exhibited a positive enrichment of the sets that group Benzopyrene-regulated (Figure 6B, upper panels) and VAF-347-regulated genes [99,101] (Figure 6B, lower panels) by GSEA, whose respective leading edges shared AhR-regulated genes (Figure 6C). Therefore, we directly evaluated the effect of 5-HT (100 μM) on the expression of representative AhR targets in the presence of an inhibitor of AhR (CH-223191) or antagonists of either 5-HT_{2B} (SB204741) or 5-HT_7 (SB269970) (Figure 6D). While 5-HT increased the expression of all assayed genes (Figure 6E), the presence of the AhR inhibitor significantly reduced the 5-HT-mediated upregulation of *CYP1B1*, *SEMA6B*, *SERPINB2* and *EREG*, but had no effect on *PDE2A*, whose 5-HT-mediated upregulation is dependent on the 5-HT_7-PKA axis [39] (Figure 6E). Importantly, the 5-HT-mediated upregulation of *CYP1B1*, *SEMA6B* and *SERPINB2* was impaired in the presence of the 5-HT_{2B} antagonist SB204741, which had no effect on the upregulation of *PDE2A* (Figure 6F). Further, the 5-HT_7 antagonist SB269970 had no effect on the expression of *CYP1B1*, but completely prevented the 5-HT-mediated upregulation of *GPR183*, *EREG* and, as expected [39], *PDE2A* (Figure 6G). Altogether, these results indicate that serotonin regulates the expression of paradigmatic AhR target genes (*CYP1B1*, *SEMA6B*, *SERPINB2*) via HT_{2B}, and also modulates the expression of additional AhR targets via HT_7.

Since the concentration of peripheral 5-HT ranges from nanomolar levels in plasma to millimolar levels in sites of inflammation, thrombus formation and fibrosis [19,105-107], we next sought to determine the effects of different 5-HT concentrations on the expression of AhR targets. As shown in Figure 7A, 5-HT dose dependently enhanced the expression of the AhR target genes (*CYP1B1*, *SEMA6B*, *AHRR*) at concentrations between 10 μM and 500 μM, while its 5-HT_7-independent effect on *PDE2A* expression was reduced at high 5-HT concentrations. The involvement of 5-HT_{2B} in the transcriptional effects of high 5-HT concentrations was confirmed through the use of the 5-HT_{2B} agonist, which significantly impaired the 5-HT-mediated upregulation of *CYP1B1*, *SEMA6B* and *AHRR* (Figure 7B). Therefore, serotonin modulates the transcriptional profile of human macrophages through the 5-HT_{2B} -dependent activation of AhR.

DISCUSSION

The polarization-specific expression of 5-HT_{2B}, an unintended target of commonly used drugs, prompted the analysis of the transcriptional consequences of 5-HT_{2B} activation in human macrophages and has led to the finding that engagement of 5-HT_{2B} by either serotonin or 5-HT_{2B} agonists shapes the macrophage transcriptome partly via activation of AhR [100]. The identification of the 5-HT-5-HT_{2B}-AhR axis supports a role for 5-HT_{2B} in myeloid cell differentiation, extends the range of cell-specific intracellular signaling pathways initiated upon serotonin receptor engagement, and illustrates a novel mechanism by which 5-HT can modulate inflammatory responses. In this regard, our results indicate that 5-HT_{2B} engagement shifts the TNF/CCL2 ratio towards the anti-inflammatory side in LPS-treated macrophages. Besides, the ability of 5-HT to modify the expression of AhR targets implies its potential capacity to modulate cellular responses towards endogenous and exogenous agents that trigger AhR activation.

Remarkably, although 5-HT and BW723C86 share the ability to activate AhR via 5-HT_{2B}, the transcriptional effects of the engagement of 5-HT_{2B} by BW723C86 on human macrophages are distinct from those triggered by 5-HT, because 5-HT can engage both 5-HT_{2B} and 5-HT_7 in human M-MØ [40]. Thus, although 5-HT_{2B} and 5-HT_7 are expressed by anti-inflammatory IL-10-producing human macrophages [40] and promote a pro-fibrotic phenotype [19,39,47,108,109], their individual engagement has different transcriptional outcomes: 5-HT_{2B} specifically promotes AhR activation whereas 5-HT_7 drives the acquisition of a PKA-dependent transcriptome [39]. Therefore, 5-HT appears to preferentially engage 5-HT_7 over 5-HT_{2B}, because the transcriptome of 5-HT-treated macrophages at early time points (6h) is predominantly shaped by 5-HT_7-initiated signals [39]. Interestingly, ligation of 5-HT_7 by 5-HT triggers protein kinase A

(PKA)-dependent signaling and transcription in macrophages [39] and other cell types [110], and results in the expression of PKA-regulated genes like *PDE2A* [39]. Since PKA activation (or increased cAMP levels) represses AhR-dependent gene expression [111,112], it is therefore possible that the binding of 5-HT to 5-HT_7, and the resulting PKA activation [39,110], might limit the activation of AhR secondary to 5-HT binding to 5-HT_{2B}. In line with this hypothesis, it is worth noting that *PDE2A* gene expression in macrophages is greatly upregulated after 5-HT binding to 5-HT_7 [39], and that PDE2A interaction with the immunophilin-like protein XAP2 (a member of the molecular complex that retains unliganded AhR in the cytoplasm) inhibits dioxin-induced AhR nuclear translocation and transcription in hepatocytes [113].

A link between 5-HT and AhR has been recently proposed in the case of intestinal epithelial cells, where 5-HT activates AhR independently on 5-HT receptors and via the serotonin transporter (SERT) [114]. The BW723C86- and 5-HT-mediated activation of AhR in macrophages that we now describe is unrelated to the 5-HT/SERT/AhR link because it is sensitive to the presence of an 5-HT_{2B} antagonist, and because M-MØ are devoid of SERT expression [115]. The existence of these two pathways (5-HT/SERT/AhR in intestinal epithelial cells and 5-HT/5-HT_{2B}/AhR in macrophages) fits with the AhR-activating ability of tryptophan catabolites [116] and supports the importance of 5-HT and related molecules in modulating inflammatory responses. Thus, and besides a receptor for environmental toxins [100], AhR mediates vascular system development, immune system polarization and resolution of inflammatory responses [117,118], processes where 5-HT_{2B} has been also implicated [16,70,71,94]. Consequently, since 5-HT_{2B} agonists (and specially BW723C86) have been widely used *in vivo* to support the participation of 5-HT_{2B} in several animal models of disease [73,74,87,88,90-92,119-121], our results suggests the possibility that AhR might contribute to the pathological effects derived from an altered expression or function of 5-HT_{2B}. Further supporting this possibility, the AhR activation

secondary to 5-HT_{2B} engagement has also been found to take place in 5-HT_{2B}^{+} HepG2 hepatoma cells. Besides, and since AhR enhances IL-10 expression and limits pro-inflammatory cytokine expression in mouse macrophages [122], and promotes immunological tolerance after macrophage capture of apoptotic cells [123], AhR might contribute to the previously reported anti-inflammatory effect of 5-HT_{2B} engagement in mouse microglia [71] and human monocytes [72].

Finally, and in spite of the ability of BW723C86 to promote AhR activation in a 5-HT_{2B}-dependent manner, it is worth noting the existence of 5-HT_{2B}-independent effects of the agonist. Specifically, the transcriptome of BW723C86-treated M-MØ includes the expression of 207 genes whose BW723C86-mediated regulation is not prevented by the 5-HT_{2B} antagonist SB204741 (Figure 1) and some of which are directly involved in osteoclastogenesis (*EGR1*, *OCSTAMP*) (Tables S2, S3). In fact, and although BW723C86 does not upregulate AhR target genes in mouse microglia, the 5-HT_{2B} agonist is capable of downregulating the expression of *Ocstamp* and *Csf1* in mouse microglia *in vitro* cultures (data not shown). Further supporting the relevance of this finding, we have later noted that the continuous presence of the 5-HT_{2B} agonist significantly impairs the expression of hallmark osteoclast markers, augments the expression of negative regulators of osteoclast differentiation, and impairs the generation of multinucleated cells during M-CSF+RANKL-driven osteoclastogenesis (data not shown). Therefore, BW723C86 impairs osteoclast differentiation in a 5-HT_{2B}-independent manner, a previously unnoticed action of this 5-HT_{2B} agonist that should be taken into account when evaluating its effects *in vivo*.

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AUTHORSHIP CONTRIBUTIONS:

CN, IR, MCE, EI, BA and CB performed research and analyzed data; CN, IR, MCE, CB, LM MAV and ALC designed the research and analyzed data; ALC wrote the paper.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. The 5-HT_{2B} agonist BW723C86 modifies the gene signature of human M-MØ through 5-HT_{2B}-dependent and independent mechanisms. **A.** Experimental design of the gene profiling experiments. **B**. Number of annotated genes whose expression is significantly (*adj* $p<0.002$) upregulated (UP) or downregulated (DOWN) in M-MØ after a 6h exposure to BW723C86 in the absence (BW *vs* DMSO) or presence of SB204741 (SB) (BW+SB *vs* SB). **C.** Number of SB204741-sensitive and SB204741-insensitive annotated genes within the group of genes significantly (*adj* $p<0.002$) regulated by BW723C86 (Genes upregulated by BW723C86, left panel; Genes downregulated by BW723C86). **D.** Volcano plot representation of the microarray data. Gene expression profiles of BW-treated M-MØ versus control M-MØ (left panel) and SB+BW-treated M-MØ versus SB-treated M-MØ (right panel) are plotted according to the $\log_2$ fold change (X axis) and $\log_{10}$ adjusted p-value (Y axis). In both plots, the shaded area includes the genes with *adj* $p<0.05$. Some of the differentially expressed genes in each case are indicated (Note the distinct relative position of *CYP1B1* and *AHRR* in both plots). **E.** Heatmap of the relative expression of the 217 genes whose significant (adj $p<0.002$) upregulation by BW723C86 (BW) is prevented in the presence of the SB204741 antagonist (SB+BW). The identity of some of the genes included within this group is indicated.

Figure 2. BW723C86 upregulates the expression of AhR target genes in a 5-HT_{2B} -dependent manner. **A.** GSEA (http://software.broadinstitute.org/gsea/index.jsp) [85] analysis of the statistics–ranked list of genes obtained from the BW723C86-treated M-MØ *versus* control M-MØ (upper panels) and BW723C86+SB204741-treated M-MØ *versus* SB204741-treated M-MØ (lower panels) limma analysis, using data sets containing the genes upregulated by Benzopyrene in human macrophages (AHR_UP_BPA_8H) or VAF-347 in human dendritic cells (VAF347_UP in DC logFC>1) [99,101]. **B**. Heatmap of the expression of four representative

AhR target genes in the transcriptome of BW723C86-treated M-MØ, SB204741-treated M-MØ and SB204741+BW723C86-treated M-MØ. **C.** Relative expression of the indicated genes in non-treated (-) and BW723C86-treated M-MØ (6h) (+) (n=7-11; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). **D.** Relative expression of *CYP1B1* and *TIPARP* in non-treated (-) and M-MØ treated with BW723C86 (+) for the indicated periods of time (n=3; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). **E.** Relative expression of *CYP1B1*, *AHRR* and *TIPARP* in non-treated (-) or M-MØ treated for 6h with BW723C86, SB204741 or both (+) (n=5; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (**C-E)** Results are shown as the expression of each gene after the different treatments and relative to its expression in control (untreated) samples. In all cases, mean and SEM are shown.

Figure 3. CYP1B1 upregulation by BW723C86 is dependent on 5-HT_{2B} and requires AhR activation. **A.** Relative expression of *CYP1B1* in non-treated (0) or M-MØ treated for 6h with the indicated concentrations of BW723C86 (n=3; *, $p < 0.05$). **B.** Relative expression of *CYP1B1* and *TIPARP* in M-MØ either untreated (-) or treated for 6h with BW723C86 (BW) in the absence or presence (+) of the AhR antagonist CH223191. M-MØ were treated in parallel with the AhR agonist FICZ (6h) for control purposes. Results are shown as the expression of each gene after the different treatments and relative to its expression in control (untreated) samples. In all cases, mean and SEM are shown (n=4; *, $p < 0.05$; ***, $p < 0.0005$). **C-D.** *AHR* mRNA (**C**) and AhR protein level (**D**) in M-MØ transfected with control siRNA (siC) or an AhR-specific siRNA (siAhR). 18 hours after transfection, M-MØ were washed and lysed for qRT-PCR (n=6; ***, $p < 0.001$) (**C**). Western blots from two independent knockdown experiments are shown in (**D**). **E**. Relative expression of *CYP1B1* and *TIPARP* in M-MØ transfected with control siRNA (siC) or AhR-specific siRNA (siAhR) and either left untreated (-) or treated for 6h with BW723C86 (+) (n=6; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). In **A-**

E, results are shown as the expression of each gene after the different treatments and relative to its expression in control (untreated) samples. In all cases, mean and SEM are shown. **F.** AhR-dependent transcriptional activity in XRE-Luc transfected M-MØ left untreated (-) or treated for 14h with BW723C86 (+) in the absence or in the presence of the AhR antagonist CH223191, added 1 hour before agonist addition. Mean and SEM of the AhR-dependent luciferase activity of five independent experiments is shown (n=5; *, $p < 0.05$; **, $p < 0.01$).

Figure 4. BW723C86 also promotes CYP1B1 upregulation and AhR activation in HepG2 cells via 5-HT_{2B}. **A.** Relative expression of *CYP1B1* in non-treated (-) or BW723C86-treated (+) HepG2 hepatoma cells exposed to the agonist for the indicated periods of time (n=3 *, $p < 0.05$; **, $p < 0.01$). Results are shown as *CYP1B1* expression in BW723C86-treated cells relative to its expression in untreated cells. In all cases, mean and SEM are shown. **B.** AhR-dependent transcriptional activity in XRE-Luc-transfected HepG2 cells left untreated (-) or treated with BW723C86 (16h) in the absence or in the presence of the indicated concentrations of the 5-HT_{2B} antagonist SB204741, added 1h before the agonist (n=4; ***, $p < 0.001$). **C**. AhR-dependent transcriptional activity in XRE-Luc-transfected HepG2 cells left untreated (-) or treated with BW723C86 (16h) in the absence or presence of the AhR antagonist CH223191, which was added 1 hour before agonist addition. HepG2 cells were treated with FICZ for control purposes (n=4; ***, $p < 0.001$). In **B-C**, results are shown as the mean and SEM of the Relative AhR-dependent luciferase activity of each sample (referred to the luciferase activity measured in untreated samples).

Figure 5. AhR activation in human macrophages is also triggered by other 5-HT_{2B} ligands and modulated by serotonin. **A.** Relative expression of *CYP1B1* in M-MØ treated for 6h with DMSO (vehicle, -), BW723C86 (+) or with increasing concentrations of APB (10 nM, 50 nM,

100 nM or 1 μM) (n=9). **B.** Relative expression of *CYP1B1* in non-treated (-) or M-MØ treated for 6h with BW723C86 (+) or with increasing concentrations of α-Methyl 5HT (AMS) (10 nM, 20 nM, 50 nM,100μM or 500 μM) (left panel) (n=6), and either in the absence or presence of SB204741 using 100μM or 500 μM AMS (right panel) (n=4). **C**. Relative expression of *CYP1B1* in M-MØ either untreated (-) or treated for 6h with 10 μM BW723C86, 5-HT (10 μM or 100 μM) or both (n=4). (**A-C)** *CYP1B1* expression after the different treatments and relative to its expression in control (untreated) samples is shown. Mean and SEM are shown ($p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 6. Serotonin upregulates AhR target gene expression in human macrophages. **A**. Heatmap of the $\log_2$ Fold Change of the expression of the indicated AhR-responsive genes in M-MØ exposed for 6 hours to BW723C86, 10 μM 5-HT or 100 μM 5-HT, as determined by RNAseq. **B**. GSEA[85] analysis of the statistics–ranked list of genes obtained from the limma analysis of the BW723C86-treated M-MØ *versus* control M-MØ (left panels), 10 μM 5-HT-treated M-MØ *versus* control M-MØ (center panels) and 100 μM 5-HT-treated M-MØ *versus* control M-MØ (right panels) transcriptomes, using data sets containing the genes upregulated by Benzopyrene in human macrophages (AHR_UP_BPA_8H) or VAF-347 in human dendritic cells (VAF347_UP in DC logFC>1) [99,101]. Normalized Enrichment Score (NES), FDR*q* and are FWER*p* values are indicated in each case. **C**. Venn diagram of the genes included within the leading edge of the GSEA panels shown (**B). D**. Schematic representation of the experiments shown in (**E-G)**. **E-G**. Relative expression of the indicated genes in non-treated M-MØ (-) or M-MØ exposed to 100 μM 5-HT (6h) in the absence (-) or presence of the AhR inhibitor CH-223191 (**E**), the 5-HT_{2B} antagonist SB204741 (**F**) or the 5-HT_7 antagonist SB269970 (**G**) (n=3 *, $p < 0.05$; **, $p < 0.01$). Results are shown as the expression of each gene after the different

treatments and relative to its expression in control (untreated) samples. In all cases, mean and SEM are shown.

Figure 7. Serotonin upregulates AhR target gene expression in human macrophages in a dose-dependent manner. **A**. Relative expression of the indicated genes in non-treated M-MØ (-) or M-MØ exposed for 6h to BW723C86 or increasing amounts of 5-HT (10-100-500 μM). **B**. Relative expression of the indicated genes in non-treated M-MØ (-) or M-MØ exposed for 6h to 500 μM 5-HT in the absence (-) or presence of the 5-HT_{2B} antagonist SB204741. Results are shown as the expression of each gene after the different treatments and relative to its expression in control (untreated) samples, and mean and SEM are shown (n=3 *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

SUPPLEMENTARY FIGURES

Figure S1. **Gene Ontology analysis of the BW723C86-treated M-MØ transcriptome. A.** Selected GSEA (http://software.broadinstitute.org/gsea/index.jsp) [85] on the "t statistic-ranked" list of genes obtained from the BW723C86-treated M-MØ versus untreated-M-MØ limma analysis, using the available data sets. The FDR*q* value is indicated in each case.

Figure S2. The 5-HT_{2B} agonist BW723C86 conditions human macrophages for altered LPS-stimulated cytokine and gene expression. **A**. Experimental design to assess the effect of BW723C86 and SB204741 on LPS-stimulated cytokine production and gene expression in M-MØ. **B.** Production of CCL2 and TNFα from LPS-stimulated (18h) M-MØ that had been non-treated (-) or exposed (6 h) to BW723C86, SB204741 or both (+), as determined by ELISA. Mean and SEM of 10/12 independent experiments are shown (*, $p < 0.05$; ***, $p < 0.001$). **C**. Expression of the indicated genes in LPS-stimulated (4h) M-MØ that had been non-treated (-) or exposed (6 h) to BW723C86, SB204741 or both (+), as determined by qRT-PCR. Results are expressed as the mRNA level of each gene relative to the level of *TBP* mRNA in the same sample. Mean and SEM of six independent experiments is shown (*, $p < 0.05$; ***, $p < 0.001$).