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Serotonin Skews Human Macrophage Polarization through HTR\textsubscript{2B} and HTR\textsubscript{7}

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Besides its role as a neurotransmitter, serotonin (5-hydroxytryptamine, 5HT) regulates inflammation and tissue repair via a set of receptors (5HT\textsubscript{1–7}) whose pattern of expression varies among cell lineages. Considering the importance of macrophage polarization plasticity for inflammatory responses and tissue repair, we evaluated whether 5HT modulates human macrophage polarization. 5HT inhibited the LPS-induced release of proinflammatory cytokines without affecting IL-10 production, upregulated the expression of M2 polarization-associated genes (SERPINB2, THBS1, STAB1, COL23A1), and reduced the expression of M1-associated genes (INHBA, CCR2, MMP12, SERPINE1, CD1b, ALDH1A2). Whereas only 5HT\textsubscript{7} mediated the inhibitory action of 5HT on the release of proinflammatory cytokines, both 5HT\textsubscript{2B} and 5HT\textsubscript{7} receptors mediated the pro-M2 skewing effect of 5HT. In fact, blockade of both receptors during in vitro monocyte-to-macrophage differentiation preferentially modulated the acquisition of M2 polarization markers. 5HT\textsubscript{2B} was found to be preferentially expressed by anti-inflammatory M2(M-CSF) macrophages and was detected in vivo in liver Kupffer cells and in tumor-associated macrophages. Therefore, 5HT modulates macrophage polarization and contributes to the maintenance of an anti-inflammatory state via 5HT\textsubscript{2B} and 5HT\textsubscript{7}, whose identification as functionally relevant markers for anti-inflammatory/homeostatic human M2 macrophages suggests their potential therapeutic value in inflammatory pathologies. The Journal of Immunology, 2013, 190: 000–000.
Additionally, 5HT functions as a regulator of inflammation and tissue regeneration and repair (14), and it modulates cytokine production in a cell type–dependent manner. In peripheral blood, serotonin is released from platelets and lymphocytes/monocytes following stimulation by LPS and IFN-γ (15), and it modulates cytokine production by myeloid cells (16). Physiologic concentrations of 5HT suppress IFN-γ–induced MHC class II expression and phagocytosis in murine macrophages (17, 18) and inhibit the LPS-induced IL-1β, IL-6, IL-12p40, and TNF-α production by human monocytes and PBMCs (19–21). In human alveolar macrophages, serotonin inhibits IL-12 and TNF-α release, but it increases IL-10, NO, and PGE2 production via 5HT2 receptors (22). In the case of dendritic cells, 5HT impairs GM-CSF/IL-4–driven human monocyte-derived dendritic cell (MDDC) differentiation by reducing costimulatory molecule and CD1a expression as well as MLR stimulatory activity while increasing CD14 expression by reducing costimulatory molecule and CD1a expression (23) and IL-10 production through 5HT1 or 5HT7 receptors (23). Others have shown that 5HT is shuttled from dendritic cells to naive T lymphocytes as a means to modulate T cell activation, proliferation, and differentiation (24), and that it might be necessary for optimal macrophage accessory function (25). In the context of inflammatory pathologies, 5HT regulates macrophage-mediated angiogenesis by reducing matrix metalloproteinase 12 (MMP12) expression in tumor-infiltrating macrophages (26), and its contribution to the development of pulmonary arterial hypertension as well as MLR stimulatory activity while increasing CD14 expression by reducing costimulatory molecule and CD1a expression (27). Thus, 5HT modulates myeloid cell functions in a variable manner, and its effects are dependent on the profile of 5HT receptors expressed by each macrophage subtype. In this regard, previous studies have reported mRNA for 5HT1E, 5HT2A, 5HT3, 5HT4, and 5HT7 in human monocytes (21), 5HT1B, 5HT1E, and 5HT2B in immature MDDC, 5HT4, and 5HT7 in mature MDDC (28), and 5HT2C in murine alveolar macrophages (29).

The differential expression of 5HT receptors in macrophages at distinct states of functional polarization prompted an evaluation of the contribution of 5HT to macrophage polarization. We now report that 5HT stimulates human macrophage polarization toward the acquisition of an M2-like phenotype, and that this effect is mediated by 5HT2B and 5HT7, which are preferentially expressed by anti-inflammatory M2 macrophages. These findings point to serotonin as a potential target for modulating macrophage polarization under physiological and pathological settings.

Materials and Methods

Generation of human monocyte-derived macrophages and cell isolation and culture

Human PBMC were isolated from buffy coats from normal donors over a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient according to standard procedures. Monocytes were purified from PBMC by magnetic cell sorting using microbeads (Miltenyi Biotech, Bergisch-Madbach, Germany). Monocytes (>95% CD14+ cells) were cultured at 0.5 × 10^6 cells/ml for 7 d in RPMI 1640 supplemented with 10% FCS (completed medium) at 37°C in a humidified atmosphere with 5% CO2 and containing GM-CSF (1000 U/ml) or M-CSF (10 ng/ml) (both from ImmunoTools, Friesoythe, Germany) to generate M1 (GM-CSF) and M2 (M-CSF) monocyte-derived macrophages, respectively. Cytokines were added every 2 d. As previously described (3, 7, 8) (Gene Expression Omnibus, accession no. GSE27792; http://www.ncbi.nlm.nih.gov/geo/), the resulting macrophage populations exhibited a differential expression of the STAB1, HTR2B, SERPINB2, COL23A1, THBS1, SERPINE1, MMP12, INHBA, CCR2, CD18, and ALDH1A2 genes (Supplemental Fig. 1A) and differed in the protein levels of FOLR2, EGLN3, and DC-SIGN (30–32) (Supplemental Fig. 1B, 1C). Before treatment with 5HT, M2 (M-CSF) macrophages were maintained in serum-free medium for 48 h, without a significant change in the level of expression of the polarization markers FOLR2, EGLN3, and DC-SIGN (Supplemental Fig. 1B, 1C). Macrophage activation was accomplished with LPS (Escherichia coli 055:B5, 10 ng/ml) for 24 h. For determination of LPS-induced cytokine expression, different doses of 5HT or the 5HT2B agonist BW727C86 (Sigma-Aldrich) were added immediately before the addition of LPS. When receptor antagonists were used (SB204741, 1 μM; SB269970, 1 μM), they were added 1 h before 5HT and LPS addition.

RNA from human liver cells was obtained as previously described (33). Murine Kupffer cells, liver sinusoidal endothelial cells (LSEC), hepatocytes, and hepatic stellate cells (Ito cells) were isolated from 12-wk-old C57BL/6 mice using sequential pronase/collagenase digestion and a Nylonoden density-gradient centrifugation as described (34). Briefly, after perfusion via portal vein with 20 ml MEM (Life Technologies BRL), liver was digested with 10 ml DMEM/F-12 (Life Technologies BRL) containing pronase (0.5 mg/g body weight; Roche Diagnostics), followed by 10 ml of DMEM/F-12 containing 7 mg collagenase (Liberase Blendzymes; Roche Diagnostics). Digested liver was flushed ex vivo with 20 ml DMEM/F-12 solution containing 0.05% pronase and 20 μg/ml DNase I (Roche Diagnostics). The resulting suspension was filtered through a filter (mesh size 70 μm) and centrifuged at 7,500 g for 15 min. The Ito cell–enriched fraction was recovered from the upper whitish layer. Kupffer and LSEC were simultaneously collected from the interface, and each cell type was further purified using anti-CD146 (for LSEC) and anti-CD11b (for Kupffer cells) microbeads (Miltenyi Biotech) according to the manufacturer’s protocol. Purity of Ito cell preparations was assessed by autofluorescence 1 d after isolation. LPS (E. coli 055:B5) was purchased from Sigma-Aldrich, and IL-10 and IL-4 were obtained from BioLegend (San Diego, CA) and used at 50 ng/ml. The monoclonal anti-human IL-10 (R&D Systems, Abingdon, U.K.) blocking Ab was added at a final concentration of 2.5 μg/ml. The 5HT2B receptor agonist BW727C86 and the antagonists SB204741 (for 5HT2B) and SB269970 (for 5HT7) were purchased from Sigma-Aldrich. Where indicated, SB204741 (1 μM) and SB269970 (1 μM) were added every 24 h during the process of in vitro macrophage generation using DMSO as a negative control.

ELISA

Macrophage supernatants were tested for the presence of cytokines and growth factors using commercially available ELISA for TNF-α, IL-10 (both from ImmunoTools), and IL-12p40 (OptiEIA IL-12p40 set; BD Pharmingen, San Diego, CA), following the protocols supplied by the manufacturers.

Reporter gene assays

The luciferase-based plasmids CRE-Luc (containing 21 cAMP-response elements), SRE-Luc (containing three serum-response elements from the c-fos promoter), and TRE-Luc (containing four copies of the canonical AP-1 binding site) were provided by Dr. W. Born (Departments of Orthopedic Surgery and Medicine, University of Zurich, Zurich, Switzerland). The TATA-pXp2 plasmid was derived from the promoterless TATA-like sequence 5′-AGGGTATATAATGGGAA′-3′ immediately upstream of the luciferase gene. The C/EBP-Luc plasmid, which contains four copies of the consensus C/EBP-binding element, was provided by Dr. Daniel G. Temsamani (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA). CHO-K1 cells stably expressing the 5HT2B receptor (ValiScreen; PerkinElmer, Boston, MA) were cultured in DMEM/F-12 with 1% dialyzed FCS and transiently transfected with 2 μg each reporter gene using SuperFect (Qiagen). After transfection, cells were cultured overnight and replated before exposure to 5HT (10−5 M) or BW727C86 (10−5 M) for 24 h. To normalize transfection efficiency, cells were cotransfected with an SV40 promoter–based β-galactosidase expression plasmid (RSV-βgal). Measurement of relative luciferase units and β-galactosidase activity were performed using a luciferase assay system (Promega) and a Galacto-Light kit (Tropix), respectively.

Quantitative real-time RT-PCR

Oligonucleotides for selected genes were designed according to the Universal ProbeLibrary system (Roche Diagnostics) for quantitative real-time PCR (qRT-PCR). Total RNA was extracted using an RNAeasy kit (Qiagen), retrotranscribed, and amplified in triplicates. Results were expressed relative to the expression level of GAPDH RNA (human samples). When indicated, results were expressed relative to the mean of the expression levels of GAPDH, TBP, H1PO1, and SDHA RNA and normalized to the values obtained in untreated cells. In the case of RNA from murine samples, qRT-PCR was done using SYBR Green I detection for quantification of the expression level of β-actin using oligonucleotides 5′-TCCTCTGTTTCTGCTGGTTTG-3′ and 5′-AGGGAAATGGCCACAGAGAT-3′.

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Confocal microscopy and immunohistochemistry

Human melanoma tissues (s.c. tissue, lymph node, and lung metastasis) were obtained from patients with primary or metastatic lesions undergoing surgical treatment. Liver biopsies were obtained from patients undergoing surgery. In all cases, samples were obtained after written informed consent and following Medical Ethics Committee procedures. Thick sections (4 μm in depth) of cryopreserved tissue were first blocked for 10 min with 1% BSA in PBS-T containing 0.1% Triton X-100, then exposed for 1 h with either a rabbit polyclonal antisera against human 5HT 2B (Santa Cruz Biotechnology), anti-CD163, anti–VE-cadherin or anti–HMB-45 mAbs, or isotype-matched control Abs. All primary Abs were used at 1–5 μg/ml, followed by incubation with FITC-labeled anti-mouse and Texas Red–labeled anti-rabbit secondary Abs. Samples were imaged with the ×63 PL-APO (numerical aperture, 1.3) immersion objective of a confocal scanning inverted AOBS/SP2 microscope (Leica Microsystems). Image processing and colocalization analyses (scatterplots) were assessed with Leica confocal software LCS-15.37. Normal human tissue microarrays were obtained from RayBiotech (Norcross, GA) and processed according to the manufacturer’s recommendations using a Prestige affinity-purified polyclonal Ab against 5HT 2B (Sigma-Aldrich) and an anti-CD68 mAb (clone PG-M1, 1/100 dilution; DakoCytomation).

Statistical analysis

Statistical significance was assessed at the 0.05 level using a paired Student t test. Where indicated, a one-way ANOVA with Newman–Keuls for multiple comparison test was done.

Results

5HT receptors are differentially expressed by M1(GM-CSF) and M2(M-CSF) macrophages

HTR2B and HTR7 genes code for the 5HT receptors 5HT 2B and 5HT 7, respectively. Gene expression profiling on pro-inflammatory M1(GM-CSF) and anti-inflammatory M2(M-CSF) macrophages (GSE27792) (8) revealed that HTR2B and HTR7 mRNA are preferentially expressed by M2(M-CSF) macrophages (Fig. 1A, 1B), and that HTR7 is the only serotonin receptor–encoding gene expressed above background levels in M1(GM-CSF) macrophages (Fig. 1A, 1B). Analysis of five independent samples confirmed that HTR2B is expressed at significantly much higher levels in M2

FIGURE 1.  Preferential expression and cytokine responsiveness of HTR2B mRNA in human M2(M-CSF) macrophages. (A) Relative expression of the genes encoding serotonin receptors in M1(GM-CSF) and M2(M-CSF) macrophages, as determined by microarray DNA analysis (GSE27792). Shown data indicate the quantile-normalized fluorescence intensity of each probe. (B) Heat map representation of the data shown in (A). (C) HTR2B mRNA expression levels in M1(GM-CSF) and M2(M-CSF) macrophages as determined by qRT-PCR (n = 5). **p < 0.005. (D) HTR2B mRNA expression levels along M2(M-CSF) macrophage polarization, as determined by qRT-PCR (n = 3). *p < 0.05 compared with the level detected in monocytes (t = 0). (E) HTR2B mRNA expression levels in M2(M-CSF) macrophages after replacement of the culture supernatant with fresh complete medium containing either M-CSF or GM-CSF for 48 h (n = 4). *p < 0.05. (F) HTR2B mRNA expression in monocytes exposed for 72 h to the indicated stimuli, as determined by qRT-PCR (n = 3). *p < 0.05 compared with the level detected in monocytes maintained in RPMI 1640 (–). (G) HTR2B mRNA expression in M2(M-CSF) macrophages exposed for 24 h to the indicated stimuli, as determined by qRT-PCR (n = 3). *p < 0.05 compared with the level detected in nonstimulated macrophages (–). (H) HTR2B mRNA expression in M2(M-CSF) macrophages exposed for 24 h to LPS in the presence of either a blocking anti–IL-10 or an isotype-matched (IgG) Ab, as determined by qRT-PCR (n = 5). *p < 0.05. **p < 0.005 after one-way ANOVA with Newman–Keuls multiple comparison test. In (C)–(H), results are presented as relative expression, which indicates HTR2B mRNA levels relative to GAPDH mRNA levels, and mean and SD are shown.

Western blot

Western blot was carried out on 10–100 μg cell lysates using standard procedures. Affinity-purified rabbit mAbs from Cell Signaling Technology (Danvers, MA) were used to detect phospho-ERK1/2 and phospho-MSK1. Detection of 5HT 2B was accomplished using a rabbit polyclonal antisera (sc-25647; Santa Cruz Biotechnology) or human vinculin (hVIN-1; Sigma-Aldrich). Detection of phospho-ERK1/2 and phospho-MSK1 was done using mouse mAbs against human α-tubulin (B-7, sc-5286; Santa Cruz Biotechnology) or human 5HT receptors in M1(GM-CSF) and M2(M-CSF) macrophages, as determined by microarray DNA analysis (GSE27792).
(M-CSF) than in M1(GM-CSF) macrophages (>500-fold) (Fig. 1C). Kinetic analysis showed that high HTR2B mRNA levels are first observed 48 h along M-CSF–driven polarization, reaching maximal levels after 96 h (Fig. 1D). Moreover, cytokine replacement experiments revealed that GM-CSF almost abrogated HTR2B mRNA levels from M2(M-CSF) macrophages (Fig. 1E). This inhibitory effect of GM-CSF prompted us to identify the cytokines affecting HTR2B expression at early time points. As shown in Fig. 1F, monocytes exposed to M-CSF for 72 h showed significantly higher levels of HTR2B mRNA than did non-stimulated monocytes, whereas other M2-polarizing cytokines (IL-4, IL-10) did not promote HTR2B mRNA expression but impaired the positive effects of M-CSF on the acquisition of HTR2B mRNA by monocytes. In fact, exposure of M2(M-CSF) macrophages to IL-4 or IL-10 for only 24 h led to a dramatic reduction in HTR2B mRNA levels, an effect also seen upon LPS stimulation (Fig. 1G). Because M2(M-CSF) macrophages respond to LPS by releasing large amounts of IL-10 (3, 8), we evaluated whether IL-10 mediated the LPS-induced downregulation of HTR2B mRNA expression. As shown in Fig. 1H, addition of a blocking anti–IL-10 Ab partly inhibited the action of LPS, indicating that IL-10 contributes to the inhibitory effect of LPS on HTR2B expression. Therefore, M2(M-CSF) macrophages are characterized by the presence of HTR2B mRNA, whose restricted expression is promoted by M-CSF. Moreover, M1(GM-CSF) macrophages generated in the presence of the Smad signaling inhibitor ALK4/5/7 exhibited an enhanced expression of HTR2B mRNA (data not shown), a result that agrees with the inhibitory action of the activin A/Smad signaling pathway on M2(M-CSF) macrophage polarization (8). Collectively, these results imply that HTR2B expression is upregulated by M-CSF but impaired by either IL-4, IL-10, GM-CSF, or ligands of ALK4/5/7.

5HT2B is expressed by M2-skewed tissue macrophages

To extend the above results, HTR2B gene expression in macrophages was assessed at the protein level. Immunocytochemical analysis confirmed the expression of 5HT2B in M2(M-CSF) (Fig. 2A), and the differential expression of 5HT2B was also seen by Western blot, which revealed a considerably higher expression in M2(M-CSF) than in M1(GM-CSF) macrophages (Fig. 2B). Next, we evaluated 5HT2B expression in macrophages from normal and pathological tissues with a predominance of M2-skewed macro-
phages. Immunohistochemical analysis indicated the colocalization of 5HT₁B and the M2-associated marker CD163 in human liver Kupffer cells (Fig. 2C). Along the same line, the 5HT₁B reactivity in lung and colon tissue greatly resembled that of the macrophage marker CD68, further supporting the expression of this serotonin receptor in tissue-resident macrophages (Supplemental Fig. 2). In line with protein data, HTR2B mRNA was detected in isolated liver Kupffer cells, with expression levels similar to those found in in vitro–generated M2(M-CSF) macrophages (Fig. 2D), whereas Htr2b mRNA expression was preferentially found in murine Kupffer cells, which showed the highest expression of the myeloid marker Emr1 (F4/80) (Fig. 2E). Taken together, these results demonstrate the expression of the 5HT₁B-coding HTR2B gene in tissue-resident macrophages with anti-inflammatory ability (alveolar, colonic macrophages) (35, 36) or whose development is dependent on M-CSF (Kupffer cells) (37).

For evaluation of the 5HT₁B expression in macrophages from a pathological setting, we analyzed tumor-associated macrophages (TAM), whose M2-skewed polarization is well established (38, 39). Analysis of cutaneous primary melanomas revealed that 5HT₁B colocalizes with CD163, a macrophage marker preferentially found in M2-polarized macrophages (30, 40), and it showed a distribution pattern different from the melanoma-specific marker HMB45 (Fig. 3A, 3B). Most 5HT₁B-positive macrophages were found in the proximity of VE-cadherin–positive endothelial cells (Fig. 3B). To confirm these results, the presence of HTR2B mRNA was analyzed in TAM isolated from carcinomas of various origins. Although to a lower extent than in vitro–generated M2(M-CSF) macrophages, HTR2B mRNA was detected in ex vivo–isolated TAM from gastric carcinoma, breast carcinoma, and melanoma (Fig. 3C). Therefore, and in agreement with its expression in M2 (M-CSF) macrophages, 5HT₁B constitutes a marker for macrophages exhibiting an M2-skewed polarization both in homeostatic and pathological conditions.

**5HT and 5HT₁B receptor engagement trigger intracellular signaling and gene expression changes in M2(M-CSF) macrophages**

To assess the functional expression of the 5HT₁B receptor in M2 (M-CSF) macrophages, we next evaluated the intracellular signals triggered in response to either 5HT stimulation or 5HT₁B engagement by the BW723C86 agonist. Screening of the activation state of 46 kinases revealed that ERK1/2 and its downstream targets MSK1/2 are phosphorylated in M2(M-CSF) macrophages in response to either 5HT stimulation or 5HT₁B ligation (data not shown). Kinetic analysis confirmed that both stimuli promote transient phosphorylation of ERK1/2 and MSK1 that peak at 10 min and diminished thereafter (Fig. 4A, 4B). Therefore, 5HT and BW723C86 activate ERK1/2 in M2(M-CSF) macrophages, confirming the presence of functional 5HT₁B receptors on their cell surface. These results are in agreement with previous results showing that ligation of the 5HT₁B receptor leads to ERK1/2 phosphorylation.
activation in cardiomyocytes and hepatic stellate cells (41, 42), and they also fit with the ability of 5HT and BW723C86 to increase the activity of ERK1/2-regulated transcription factors that control macrophage activation and polarization (AP-1, C/EBP, SRF) (Supplemental Fig. 3).

The signaling capability of the 5HT2B receptor in macrophages led us to explore whether 5HT or 5HT2B ligation could modulate macrophage polarization. To that end, the expression of polarization-specific markers (8) was monitored in M2(M-CSF) macrophages that had been exposed to either 5HT or BW723C86 for 24 h. The expression of the M1(GM-CSF) polarization markers ALDH1A2, CD1B, and MMP12 was significantly reduced by either 5HT or BW723C86 (Fig. 4C, Supplemental Fig. 4), whereas both agents enhanced the expression of the M2(M-CSF)–specific markers SERPINB2, COL23A1, THBS1, and STAB1 (Fig. 4C). Besides, 5HT, but not the agonist, reduced the expression of the M1(GM-CSF)–specific markers INHBA, CCR2, and SERPINE1 (Supplemental Fig. 4). These results are in agreement with the reported ability of 5HT to reduce the expression of MMP12 in murine macrophages (26), and they demonstrate that both 5HT and the 5HT2B agonist BW723C86 influence the phenotypic macrophage polarization.

Because the defining difference between M1(GM-CSF) and M2(M-CSF) macrophages that had been maintained for 48 h in serum-free medium were exposed to LPS in the presence of 5HT or the 5HT2B agonist BW723C86. Each determination was performed in triplicate on six independent samples, and means and SD are shown (n = 6). *p < 0.05 compared with the cytokine levels in LPS-treated macrophages.

**FIGURE 4.** Effects of 5HT and the 5HT2B agonist BW723C86 on intracellular signaling, polarization marker expression, and cytokine production. (A) ERK1/2 phosphorylation in lysates of untreated (–), 10^{-2} M 5HT-treated, or 10^{-2} M BW723C86-treated M2(M-CSF) macrophages, as determined by Western blot using Abs specific for total or phosphorylated ERK1/2 (upper panels). The relative level of phosphorylated ERK1/2 was determined after densitometric analysis and normalization with α-tubulin levels and is referred to the level of ERK1/2 phosphorylation in untreated cells (–) (lower panel). One representative experiment out of three different donors and experiments is shown. (B) MSK1 phosphorylation in lysates of untreated (–), 10^{-2} M 5HT-treated, or 10^{-2} M BW723C86-treated M2(M-CSF) macrophages, as determined by Western blot using Abs specific for phosphorylated MSK1 (upper panels). The relative level of phosphorylated MSK1 was determined after densitometric analysis and normalization with α-tubulin levels and is referred to the level of MSK1 phosphorylation in untreated cells (–) (lower panel). One representative experiment out of two different donors and experiments is shown. (C) ALDH1A2, COL23A1, STAB1, SERPINB2, and THBS1 mRNA expression levels in M2(M-CSF) macrophages exposed to either 5HT or BW723C86 (10^{-2} M) for 24 h, as determined by qRT-PCR. Results are expressed as relative expression (relative to TBP, HPRT1, SDHA, and GAPDH RNA levels) and are referred to the expression level observed in the absence of stimulation (–). Mean and SD of triplicate determinations on 10 (for 5HT) or 13 independent samples (for BW723C86) are shown. *p < 0.05, **p < 0.005. (D) ELISA determination of TNF-α, IL-12p40, and IL-10 levels in culture supernatants of M2(M-CSF) macrophages untreated or stimulated with LPS (10 ng/ml) for 24 h in the absence or presence of the indicated concentrations of 5HT or the 5HT2B agonist BW723C86. Each determination was performed in triplicate on six independent samples, and means and SD are shown (n = 6). *p < 0.05 compared with the cytokine levels in LPS-treated macrophages.
whether additional 5HT receptors might contribute to the macrophage polarization skewing ability of 5HT. As predicted from the gene expression profiling data (Fig. 1A, 1B), M2(M-CSF) macrophages also contained higher levels of HTR7 mRNA than did M1(GM-CSF) macrophages (Fig. 5A). Similar to HTR2B, HTR7 mRNA expression was detected at early time points (Fig. 5B) and inhibited by GM-CSF (Fig. 5C), IL-4, or LPS (Fig. 5D, 5E). However, and unlike HTR2B, HTR7 mRNA expression was not significantly affected by M-CSF, IL-10 (Fig. 5D, 5E), or inhibition of the M-CSF–dependent ERK1/2 activation (data not shown). Therefore, we next determined whether HTR7 contributed to the modulation of gene expression and cytokine profile promoted by 5HT. To that end, and owing to the lack of specific 5HT7 agonists, 5HT-mediated changes were monitored in the presence of the 5HT7 receptor–specific antagonist SB269970 and using the 5HT2B–specific antagonist SB204741 as a control. Whereas the 5HT7 antagonist SB269970 prevented the 5HT-induced upregulation of STAB1, SERPINB2, THBS1, and COL23A1 gene expression (Fig. 6A), the 5HT2B antagonist SB204741 only inhibited the positive effect of 5HT on STAB1 and SERPINB2 gene expression (Fig. 6A). Therefore, the modulation of the macrophage polarization phenotype caused by 5HT in M2(M-CSF) macrophages is mediated by 5HT2B and HTR7.

5HT abrogates proinflammatory cytokine production in macrophages through HTR7.

Because 5HT2B did not mediate the inhibitory action of 5HT on the LPS-stimulated release of IL-12p40 and TNF-α from M2(M-CSF) macrophages (Fig. 4D), the potential role of 5HT7 was tested. The presence of the 5HT7 antagonist SB269970 significantly reverted the inhibitory effect of 5HT on the LPS-stimulated production of both TNF-α and IL-12p40 (Fig. 6B). Conversely, in agreement with the results observed with the 5HT2B agonist BW723C86, the 5HT2B antagonist SB204741 did not affect the inhibitory effect of 5HT on the LPS-stimulated production of TNF-α and IL-12p40 from M2(M-CSF) macrophages that had been maintained for 48 h in serum-free medium (Fig. 6B). Because none of the antagonists modified the basal level of cytokine production (Fig. 6B), these results indicated that 5HT modulates the cytokine profile of LPS-stimulated human M2(M-CSF) macrophages primarily through interaction with the 5HT7 receptor.

**SHT influences macrophage polarization**

Given the ability of 5HT to alter phenotypic and functional macrophage polarization, we hypothesized that it might also contribute to the cytokine-dependent polarization of monocytes. To test this hypothesis, monocytes were subjected to M-CSF–driven polarization in the presence or absence of 5HT2B or 5HT7 antagonists, and the expression of M1- and M2-specific markers was measured in the resulting monocyte-derived macrophage populations. Regarding M2(M-CSF) markers, the expression of SERPINB2 and HTR2B mRNA was significantly diminished in the presence of the 5HT2B antagonist SB204741, whereas STAB1 and THBS1 expression was significantly reduced in the presence of the 5HT7 antagonist SB269970 (Fig. 7A). Moreover, the simultaneous presence of both inhibitors limited the expression of COL23A1 (Fig. 7A). Alternatively, the 5HT2B antagonist SB204741 alone impaired the expression of the M1(GM-CSF) markers ALDH1A2 and CCR2 mRNA, whereas the combined presence of SB204741 and SB269970 reduced the expression of MMP12 and enhanced that of CD16B mRNA (Fig. 7B). Collectively, these results demonstrate that antagonizing 5HT2B or 5HT7 along the monocyte-to-macrophage differentiation process alters the pattern of expression of polarization markers, thus demonstrating that serotonin contributes to the acquisition of macrophage polarization markers during the M-CSF–driven monocyte-to-macrophage transition.
or 5HT plus the 5HT 7 antagonist SB269970 (SB-7), the 5HT 2B antagonist stimulated with LPS (10 ng/ml) for 24 h in the absence or presence of 5HT, levels in culture supernatants of M2(M-CSF) macrophages untreated or antagonists. (*p < 0.005 compared with the level of expression detected in the absence of antagonists.

Effects of 5HT receptor antagonists on the 5HT-dependent changes in the gene expression and LPS-induced cytokine profile of M2 (M-CSF) macrophages. (A) STAB1, SERPINB2, COL23A1, THBS1, and ALDH1A2 mRNA expression levels in M2(M-CSF) macrophages exposed for 24 h to 5HT (10^{-5} M) in the absence or in the presence of SB269970 (SB-7), SB204741 (SB-2B), or both, as determined by qRT-PCR. Results are expressed as relative expression and are referred to the expression level of each gene in the presence of 5HT. Mean and SD of triplicate determinations on three independent samples are shown. *p < 0.05, **p < 0.005 compared with the level of expression detected in the absence of antagonists.

FIGURE 6. ELISA determination of TNF-α, IL-12p40, and IL-10 levels in culture supernatants of M2(M-CSF) macrophages untreated or stimulated with LPS (10 ng/ml) for 24 h in the absence or presence of 5HT, or 5HT plus the 5HT7 antagonist SB269970 (SB-7), the 5HT2B antagonist SB204741 (SB-2B), or both antagonists. Each determination was performed in triplicate on five independent samples, and mean and SD are shown. *p < 0.05, **p < 0.005 compared with the level of each cytokine in macrophages exposed to LPS plus 5HT.

Discussion

Although 5HT controls important functions in the CNS, recent advances have now shown that 5HT functions as a regulator of cell proliferation as well as inflammation, tissue regeneration, and repair (12, 42, 43). The importance of macrophage polarization for an adequate regulation of these latter processes justifies the presence of functional 5HT receptors on their cell membrane and the 5HT ability to modulate macrophage effector functions. However, 5HT effects on the immune system are pleiotropic and incompletely understood (14). In the case of myeloid cells, 5HT variably affects the production of proinflammatory and anti-inflammatory cytokines from monocytes, macrophages, and dendritic cells, mainly because of the existence of a large set of 5HT receptor subtypes that exhibit cell- and tissue-specific patterns of expression (44). In the present study we provide evidence for the preferential expression of 5HT2B in anti-inflammatory macrophages and liver Kupffer cells, demonstrate the effects of 5HT on the phenotypic and functional human macrophage polarization, and identify 5HT2B and 5HT7 as the serotonin receptors that mediate the polarization skewing action of 5HT at the gene expression (5HT2B and 5HT7) and functional (5HT7) levels.

Kupffer cells represent >50% of resident macrophages in the whole body, and their development and maturation are dependent on M-CSF (37). Because of their location in the liver sinusoids, Kupffer cells are continuously exposed to the 5HT levels found in peripheral blood after its release from either enterochromaffin cells (under physiological conditions) or platelets (after degranulation events). Therefore, it is conceivable that 5HT2B might contribute to adjusting the activation state of Kupffer cells to extracellular 5HT levels. Additionally, because 5HT2A and 5HT2B mediate serotonin-dependent liver regeneration (12), a process where Kupffer cell–derived cytokines also participate (45), it is tempting to speculate that the presence of 5HT2B on Kupffer cells has a direct impact on liver regeneration. In this regard, the ability of the 5HT2B agonist BW723C86 to favor the in vitro maintenance of the M2 macrophage polarization state, characterized by its tissue repair– and cell growth–promoting properties, supports such a hypothesis.

Besides Kupffer cells, we have identified 5HT2B expression in TAM, which display a tumor-promoting and immunosuppressive M2-like polarization (46). A link between 5HT and macrophages in cancer progression has been already established, as 5HT enhances angiogenesis in murine colon cancer allografts via the 5HT-dependent reduction of MMP12 expression by tumor-infiltrating macrophages (26). Our results on the phenotypic changes induced by 5HT and BW723C86 on human macrophages are compatible with these findings because: 1) both stimuli inhibit MMP12 mRNA expression (Supplemental Fig. 4), thus suggesting 5HT2B as a mediator of inhibitory effect of 5HT on MMP12 expression; and 2) both stimuli skew macrophages toward M2 polarization, which is associated with potent proangiogenic activity (46). Therefore, our results suggest that 5HT2B is a relevant receptor whose ex-
pression on human macrophages might mediate the proliferative and tissue repair activity of 5HT in peripheral tissues. Alternatively, the location of 5HT2B-expressing TAM in the proximity of VE-cadherin–positive endothelial cells (Fig. 3) might be also of significance considering the link between 5HT2B expression and pulmonary arterial hypertension (PAH) (47). PAH is characterized by vascular remodeling secondary to abnormal cell proliferation and extracellular matrix deposition, with the latter two activities commonly linked to M2 macrophage polarization. Thus, the presence of 5HT2B in lung macrophages, as well as its expression in human TAM, which promote tumor cell growth and dissemination, raises the question of whether 5HT2B-positive macrophages might contribute to PAH by enhancing proliferation of other cell types (endothelium, smooth muscle) or through exaggerated extracellular matrix deposition. This possibility is compatible with the recent finding that PAH development requires the expression of the murine 5HT2B receptor in bone marrow–derived progenitor cells (27).

Previous reports have documented the ability of 5HT to modulate the production of numerous cytokines in mononuclear cells, monocytes, monocyte-derived dendritic cells, and ex vivo–isolated macrophages (14, 48). Whereas the inhibition of TNF-α production is a general finding, the effects of 5HT on other proinflammatory cytokines and IL-10 are variable, and they appear to be cell type–dependent. Interestingly, most previous studies have evaluated 5HT on myeloid cells in medium containing serum, where 5HT concentration is as high as 1–2 μM, thus potentially leading to an underestimation of the influence of 5HT. Alternatively, the presence of serum factors could modulate or synergize with 5HT, and it might have caused an overestimation of the 5HT influence. In an effort to avoid these potentially confounding issues, we have carried out all experiments on macrophages maintained in serum-free medium for 48 h. Thus, apart from the cell type–specific nature of 5HT actions, the distinct medium used for assaying 5HT might explain the discrepancy between the lack of effect of 5HT on IL-10 release seen in our experiments and those of others (20, 49) and the positive action of 5HT in IL-10 production described in human alveolar cells and monocyte-derived dendritic cells (22, 23). Despite these discrepancies, our results are in agreement with the lack of effect of 5HT1 receptors on the LPS-induced monocyte cytokine production (21).

The results presented in this study reveal that the modulation of the phenotypic and functional macrophage polarization by 5HT is mediated by both 5HT2B and 5HT7 receptors. At the functional level, our results point to 5HT7 as the critical receptor mediating the effect of 5HT on macrophage cytokine production. Within the myeloid cell lineage, HTR7 mRNA has been identified in human monocytes (21) and mature MDDC (28), and, similar to the case of M2(M-CSF) macrophages, 5HT7 has been shown to negatively regulate the secretion of TNF-α from mature MDDC (28) and LPS-activated monocytes (21). Whereas 5HT7 agonists also enhance the release of IL-1β and IL-8 from MDDC (28) and monocytes (21), opposite activities on IL-12 have been reported in both cell types. Our results extend those previous findings to the case of anti-inflammatory macrophages and, to our knowledge, also provide the first evidence that 5HT7 directly contributes to the gene expression changes provoked by 5HT in human macrophages.

The ability of 5HT to promote the maintenance of the M2 phenotype and to impair the release of proinflammatory cytokines is in agreement with the recent finding that platelet-derived 5HT delays activated virus-specific CD8+ T cell infiltration in a murine model of noncytotoxic lymphocytic choriomeningitis viral infection (50), and suggests that tissue macrophages might overcome the action of peripheral 5HT to display their full range of proinflammatory activities. In fact, note that 5HT is released from enterochromaffin cells along the gut, where macrophages mostly release anti-inflammatory molecules (including IL-10) upon stimulation (36). 5HT might therefore contribute to the maintenance of the anti-inflammatory profile of lamina propria macrophages, an effect that could be mediated by 5HT7 on intestinal macrophages (Supplemental Fig. 2). This possibility, combined with the functions of 5HT in the hepato-gastrointestinal tract (9), should prompt further investigation on the role of the macrophage 5HT2B and 5HT7 receptors in liver and gut physiopathology.

Disclosures

The authors have no financial conflicts of interest.

References

serotonin skews macrophage polarization


Supplementary Figure 1. - A. - Relative mRNA expression levels of the indicated genes in four independent M1(GM-CSF) and M2(M-CSF) macrophage samples, as determined by qRT-PCR. Results are shown as the log of the ratio between the expression of each mRNA in M2(M-CSF) and M1(GM-CSF) macrophages. B,C. - Expression of FOLR2 (B), DC-SIGN (C) and EGLN3 (C) in M1(GM-CSF), M2(M-CSF) and M2(M-CSF) macrophages that had been maintained under serum-free conditions for 48 hours (M2*), as determined by flow cytometry with a FOLR2-specific polyclonal antiserum (B) or Western blot using polyclonal antisera specific for either DC-SIGN or human PHD3 (C). In C, and for loading control purposes, the levels of GAPDH were determined with a monoclonal antibody against GAPDH (sc-32233; Santa Cruz Biotechnology, Santa Cruz, CA). In B-C, shown are the representative results of one out of two independent experiments.
Supplementary Figure 2.- Expression of 5-HT2B in human tissues, as determined by immunohistochemistry on lung (a,b), colon (c,d) and liver (e,f) tissue sections. Shown are light microscopy images (40X magnification) of the indicated tissues after staining with an anti-5-HT2B antibody rabbit polyclonal (a,c,e) or an anti-CD68 murine monoclonal antibody (b,d,f). Similar results were obtained on two independent samples of each tissue, and one of them is shown.
Supplementary Figure 3.- Activity of the indicated reporter constructs in CHO-K1 (5-HT$_{2B}$) stable transfectants either unstimulated (−) or exposed to 5-HT or BW723C86 (10$^{-5}$ M). For normalization, cells were co-transfected with the RSV-β-gal expression plasmid, and results are presented as Relative Promoter Activity, that indicates the units of luciferase activity per unit of β-galactosidase activity for each assay condition. Three independent experiments were done, and evaluation of luciferase activity was done in triplicate. Shown are the mean and standard deviations of the three experiments (*, p < 0.05).
Supplementary Figure 4.- Effects of 5-HT and BW723C86 on polarization marker expression. SERPINE1, MMP12, INHBA, CCR2 and CD1B mRNA expression levels in M2(M-CSF) macrophages exposed to either 5-HT or BW723C86 (10^{-5} M) for 24 hours, as determined by qRT-PCR. Results are expressed as Relative Expression (relative to TBP, HPRT1, SDHA and GAPDH RNA levels) and referred to the expression level observed in the absence of stimulation (-). Mean and standard deviation of triplicate determinations on ten (for 5-HT) or thirteen independent samples (for BW723C86) are shown (*, $p < 0.05$; **, $p < 0.005$).