The Serotonergic Receptors of Human Dendritic Cells: Identification and Coupling to Cytokine Release

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The Serotoninergic Receptors of Human Dendritic Cells: Identification and Coupling to Cytokine Release

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The neurotransmitter 5-hydroxytryptamine (5-HT), commonly known as serotonin, is stored at peripheral sites in mast cells and released from this peripheral source upon IgE cross-linking. In this study, we investigated the expression of serotoninergic receptors (5-HTR), the signaling pathway, and biological activity of 5-HT on human dendritic cells (DC), showing that immature and mature DC expressed mRNA for different serotoninergic receptors. Thereby, the mRNA of 5-HTR1B, 5-HTR1F, 5-HTR2A, and 5-HTR2E, one splicing variant of the 5-HTR2A, 5-HTR4, and 5-HTR4 receptors were detected. Immature DC preferentially expressed mRNA for the heptahelial 5-HTR1B, 5-HTR1F, and 5-HTR2E receptors, while mature DC mostly expressed 5-HTR4 and 5-HTR7. The mRNA expression level of the ligand-gated cation channel 5-HTR3 and the heptahelial 5-HTR2A did not significantly change during maturation. Isotype-selective receptor agonists allowed us to show that 5-HT stimulated 5-HTR3-dependent Ca2+ influx in immature and mature DC. Moreover, we revealed that 5-HTR4 and 5-HTR7 receptor stimulation induced intracellular Ca2+ mobilization via G11 proteins in immature, but not mature, DC. Activation of 5-HTR4 and 5-HTR7 induced cAMP elevation in mature DC. Functional studies indicated that activation of 5-HTR4 and 5-HTR7 enhanced the release of the cytokines IL-1β and IL-8, while reducing the secretion of IL-12 and TNF-α in mature DC. In summary, our study shows that 5-HT stimulated, in a maturation-dependent manner, different signaling pathways in DC. These data point to a role for 5-HT in regulating the immune response at peripheral sites. The Journal of Immunology, 2004, 172: 6011–6019.

Serotonin (5-hydroxytryptamine (5-HT)) is a well-characterized neurotransmitter and vasoactive amine involved in the regulation of a large number of physiological functions such as sleep, appetite, and behavior (1,2). 5-HT has also immunomodulatory effects by regulating a wide variety of cell responses such as migration, phagocytosis, superoxide anion generation, and cytokine production (3–6). 5-HT is released at inflammatory sites by IgE-activated mast cells and platelets (7), and recent findings point to a role of 5-HT in the pathophysiology of asthma (8). The wide variety of 5-HT-mediated functions is paralleled by the high pharmacological complexity of responses due to existence of different classes of serotoninergic receptors (5-HTR) (9). The 5-HTR class consists of at least five subtypes named 5-HTR1A, 5-HTR1B, 5-HTR1D, 5-HTR1F, and 5-HTR1A interacts with several G proteins eliciting different responses (10–12). 5-HTR1B and 5-HTR1D are coupled to formation of inositol phosphates through interaction with pertussis toxin-sensitive Gi/o and pertussis toxin-insensitive Gα15 proteins (12,13). The G protein-coupled 5-HTR3 class includes three different subtypes: 5-HTR2A, 5-HTR2B, and 5-HTR2C (14–17). 5-HTR4 receptors are ligand-gated cation channels triggering depolarization of the plasma membrane through activation of Na+ and K+ fluxes (18). The 5-HT5A receptor has two splice variants (5-HT5A1 and 5-HT5A2) (19,20). The heptahelial 5-HT5A subtype is less characterized (21). 5-HT5A and 5-HT5B are linked to Gs protein-mediated stimulation of adenyl cyclase (22,29).

Dendritic cells (DC) are APCs specialized in activating naive T lymphocytes to initiate primary immune responses (30,31). DC originate from hemopoietic stem cells that migrate into target sites to capture Ags. During circulation through the body DC undergo maturation, a process that entails acquisition of high levels of surface MHC and costimulatory molecules, expression of different chemokines, and production of cytokines. DC migrate to secondary lymphoid organs where they play a crucial role in the development of Th1/Th2-modulated immune responses through release of cytokines and chemokines (32). DC also produce several proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 that profoundly affect the outcome of inflammatory reactions (33). In this study, we characterized the biological activity of 5-HT in DC, showing that 5-HT-mediated responses depend on the differentiation stage of DC. 5-HT induced Ca2+ mobilization from intracellular stores in immature, but not in LPS-matured, DC. On the contrary, 5-HT triggered, in mature DC, Ca2+ influx through the plasma membrane, cAMP increase, IL-1β and IL-8 release, while it reduced secretion of IL-12 and TNF-α.
Materials and Methods

Reagents

5-HT, 5-methoxytryptamine (2-MHT), N-methyl-5-HT (2Me5HT), R(-)-DOI-hydrochloride (DOI), ketanserin, recombinant human complement fragment 5a (C5a), pertussis toxin (PTX), and lysophosphatidylcholine were obtained from Sigma-Aldrich (Deisenhofen, Germany); 5-carboxamidotryptamine maleate (5-CT), BRL-54443, 8-hydroxy-DPAT-hydrobromide (8-HDPAT), aniptoline hydrochloride (AnHCL), pimozone, RS-39604 hydrochloride, and SB-269970 hydrochloride were purchased from Tocris (Bristol, U.K.). Macrophage inflammatory protein-3/chemokine ligand 19 (MIP-3β/CCL19) from PeproTech (London, U.K).

Preparation of human DC

Peripheral mononuclear cells were separated from buffy coats using a Ficoll gradient. After separation, the leukocyte-containing pellet was resuspended in 2 ml of PBS containing 0.15% EDTA and 0.5% BSA. Cells were separated with anti-CD14 mAb-coated MicroBeads using Macs single use separation columns from Miltenyi Biotec (Bergisch Gladbach, Germany).

FIGURE 1. Immature and mature DC express the mRNA for several 5-HTR subtypes (A and B). RT-PCR analysis was performed with mRNA isolated from purified DC incubated in the absence (immature) or presence (mature) of LPS for 24 h (see Materials and Methods). A, Lanes: A, 5-HT2B; B, 5-HT2A; C, 5-HT1B; D, 5-HT1B. B, Lanes: E, 5-HT3; F, 5-HT4; G, 5-HT6. One representative experiment of four is shown (n = 4). C and D, Relative quantification of the bands was performed by iCycler as described in Materials and Methods. Data are means ± SEM (n = 4). Global differences between groups: p ≤ 0.0001 (ANOVA); ≤0.001 (**), p ≤ 0.01 (**); p ≤ 0.05 (*) compared with untreated cells (Tukey’s multiple comparison test).

FIGURE 2. 5-HT triggers Ca²⁺ transients in immature and mature DC. Immature DC (A) or mature DC (B) were loaded with the Ca²⁺ indicator flura-2/AM and stimulated with the indicated 5-HT concentrations. Representative traces are shown. Experiments were repeated five times with similar results.
CD115\textsuperscript{high} and are also referred to as immature DC. Maturation of DC was induced by a 24 h incubation in the presence of 3 μg/ml LPS (LPS; Sigma-Aldrich). Mature DC were >95% CD80\textsuperscript{high}, CD86\textsuperscript{high}, CD83\textsuperscript{high}, and CD115\textsuperscript{low}. mAbs and their respective isotype controls were from Coulter-Immunotech (Krefeld, Germany).

Detection of 5-HTR mRNA by RT-PCR analysis

The mRNA was isolated with QIAshredder and RNeasy kits (Qiagen, Hilden, Germany). mRNA, Moloney murine leukemia virus reverse transcriptase and pd(N)\textsuperscript{6} primers (Life Technologies, Gaithersburg, MD) were used to obtain cDNA. All oligonucleotides used as primers in PCR were designed to recognize sequences specific for each target cDNA. Primer sequences are as follows: 5-HTR\textsubscript{1A} (411-bp product): sense: 5'-GCC GCG TGC GCT CAT CTC G-3', antisense: 5'-GCG GCG CCA TCG TCA CCT T-3'; 5-HTR\textsubscript{1B} (460-bp product): sense: 5'-CAG CGC CAA GGA CTA CAT TTA CCA-3', antisense: 5'-GAA GAA GGG CGG CAG CGA GAT AGA-3'; 5-HTR\textsubscript{1E} (461-bp product): sense: 5'-CAG CGC CAA GGA CTA CTA TTA CCA-3', antisense: 5'-GAA GAA GGG CGG CAG CGA GAT AGA-3'; 5-HTR\textsubscript{2A} (359-bp product): sense: 5'-ACT CGC CGA TGA TAA CTT TGT CCT-3', antisense: 5'-TGA CGG CCG ATG CTG AAC G-3'; 5-HTR\textsubscript{2B} (416-bp product): sense: 5'-GGC CCC TCC CAC TTC CCG TTG TTC T-3', antisense: 5'-TAG GCC TTT ATT TGT TTT GTG TGT T-3'; 5-HTR\textsubscript{2C} (449-bp product): sense: 5'-TGT GCC CCG TCT GGA TTT CTT TAG-3', antisense: 5'-CTC TCG GCC TCA CCA TCA CCA CCA-3'; 5-HTR\textsubscript{3} (448/352 bp): sense: 5'-CCG GCC GGC ATG CTG AAC G-3', antisense: 5'-GCC CGC GCC CAC AAG GAC AAA AG-3'; 5-HTR\textsubscript{4} (436 bp): sense: 5'-GCC CTG GCC GGC CCA TCA CCG CCA-3', antisense: 5'-GGA GAA GGG CGG CAG CGA GAT AGA-3'; 5-HTR\textsubscript{5} (436 bp): sense: 5'-GCC CTG GCC GGC CCA TCA CCG CCA-3', antisense: 5'-GGA GAA GGG CGG CAG CGA GAT AGA-3'; 5-HTR\textsubscript{6} (342 bp): sense: 5'-GCC CCG GCC ATG CTG AAC G-3', antisense: 5'-GGA GAA GGG CGG CAG CGA GAT AGA-3'; 5-HTR\textsubscript{7} (436 bp): sense: 5'-GCC CTG GCC GGC CCA TCA CCG CCA-3', antisense: 5'-GGA GAA GGG CGG CAG CGA GAT AGA-3'; 2-microglobulin (259 bp): sense: 5'-GCC CTG GCC GGC CCA TCA CCG CCA-3', antisense: 5'-GGA GAA GGG CGG CAG CGA GAT AGA-3'.

Thirty PCR cycles were run at 94°C (denaturation, 1 min), 62°C (annealing, 1 min), and 72°C (extension, 1 min). The generated products were subjected to electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Intensity of the different bands in PCR gels was quantified by measuring the OD with a OneDscan computer software package (Scanalytics, Fairfax, VA). The cDNA amplification was linear in an amplification range of 24–34 cycles. The identity of the PCR products was confirmed by sequencing after cloning using pCRII vectors. Controls run without reverse transcriptase yielded no PCR products.

**FIGURE 3.** 5-HTR\textsubscript{1} and 5-HTR\textsubscript{2} agonists trigger Ca\textsuperscript{2+} transients in immature, but not mature, DC. Immature (A–D) or mature DC (E) were loaded with fura-2/AM and stimulated with increasing concentrations of the reported agonists. Representative curves are shown. Experiments were repeated five times with similar results.
Quantification by real-time PCR

Total RNA was extracted using the RNeasy kit according to the manufacturer’s protocol (Qiagen). Briefly, DNase I (Invitrogen, San Diego, CA) treatment, 1 μg of total RNA from each sample, was used as a template for the reverse transcription reaction. Fifty microliters of cDNA were synthesized using M-MLV reverse transcriptase and pd(N)6 primers (Life Technologies). All samples were reverse transcribed under the same conditions (25°C for 10 min, 48°C for 30 min) and from the same reverse transcription master mix to minimize differences in reverse transcription efficiency. All oligonucleotide primers for real-time PCR were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3www.cgi) and synthesized by Invitrogen.

For iCycler reaction, a master mix of the following compounds was prepared to the indicated end concentration: 10 μl of SYBR Green master mix (Bio-Rad, Hercules, CA), 6 μl of water, 1 μl of sense and 1 μl of antisense primers (500 nM). This master mix (18 μl) was filled in the iCycler strips and 2 μl of cDNA (0.625, 2.5, 10, or 40 ng reverse-transcribed total RNA) was added as PCR template. The following iCycler experimental run protocol was used: denaturation program (95°C for 9 min), amplification, and quantification program repeated 40 times (95°C for 30 s, 60°C for 30 s, 72°C for 30 s), melting curve program (60–95°C with a heating rate of 0.1°C per second), and finally a cooling step to 4°C. Emission fluorescence for each reaction was measured during the extension phase. Real-time PCR efficiency (E) was calculated from the given slopes, with the iCycler software, as previously described (34).

The cycle threshold (CT), i.e., the cycle number at which the amount of the amplified gene reaches threshold fluorescence, was determined using the iCycler software. The relative expression ratio (R) of the different target genes was calculated based on efficiency (E) and cycle threshold (CT), deviation of an unknown sample vs a control, and compared with the housekeeping gene GAPDH, as previously described (34, 35).

Intracellular Ca\(^{2+}\) measurement

Ca\(^{2+}\) transients were measured in DC loaded with the Ca\(^{2+}\) indicator fura-2/AM (Calbiochem, La Jolla, CA) by using the digital fluorimeter unit Atto (AM) (Calbiochem, La Jolla, CA) treatment, 1 μg of total RNA from each sample, was used as a template for the reverse transcription reaction. Fifty microliters of cDNA were synthesized using M-MLV reverse transcriptase and pd(N)6 primers (Life Technologies). All samples were reverse transcribed under the same conditions (25°C for 10 min, 48°C for 30 min) and from the same reverse transcription master mix to minimize differences in reverse transcription efficiency. All oligonucleotide primers for real-time PCR were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3www.cgi) and synthesized by Invitrogen.

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Intracellular Ca\(^{2+}\) measurement

Ca\(^{2+}\) transients were measured in DC loaded with the Ca\(^{2+}\) indicator fura-2/AM (Calbiochem, La Jolla, CA) by using the digital fluorimeter unit Atto (Zeiss, Oberkochen, Germany). Briefly, DC were incubated with 2 μM fura-2/AM for 30 min at 37°C in a Ca\(^{2+}\)-free Hank’s BSA solution. Cells were then washed twice and finally resuspended in the same buffer containing 1.5 mM CaCl\(_2\) and MgCl\(_2\). Traces were followed spectrofluorometrically and Ca\(^{2+}\) transients were determined by multiple cell acquisitions with the 340/380 wavelength excitation ratio at an emission wavelength of 505 nm. Curves shown are representatives of the whole cell population.

Cytokine assays

IL-8 was measured in DC supernatants by ELISA (BD PharMingen, San Diego, CA). IL-1β was determined by using ELISA kits from Amersham Pharmacia Biotech (Piscataway, NJ). IL-12 and TNF-α present in DC supernatants were measured by ELISA using matched pair mAbs from R&D Systems (Abingdon, U.K.). Samples were assayed in triplicate for each condition.

Measurement of intracellular cAMP

Intracellular cAMP levels were determined by an enzyme immunoassay (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Forskolin (Sigma-Aldrich) was used as a positive control. cAMP transients were measured in DC loaded with the Ca\(^{2+}\) indicator fura-2/AM for 30 min at 37°C in a Ca\(^{2+}\)-free Hank’s BSA solution. Cells were then washed twice and finally resuspended in the same buffer containing 1.5 mM CaCl\(_2\) and MgCl\(_2\). Traces were followed spectrofluorometrically and Ca\(^{2+}\) transients were determined by multiple cell acquisitions with the 340/380 wavelength excitation ratio at an emission wavelength of 505 nm. Curves shown are representatives of the whole cell population.

Statistical analyses

Unless stated otherwise, data are expressed as mean ± SEM. ANOVA was used to compare experimental groups to control values. When the global test of differences was significant at the 5% level, pairwise tests of differences between groups were applied (Tukey’s comparison test). For PCR bands, statistical analysis was performed by the Dunnet comparison test (ANOVA).

Results

Human DC express mRNA for different 5-HTR subtypes

Expression of mRNA for the different 5-HTR subtypes was analyzed by RT-PCR in immature and mature DC. Fig. 1A shows that immature and LPS-matured DC expressed mRNA for 5-HTR\(_{1A}\), 5-HTR\(_{1E}\), 5-HTR\(_{2A}\), and 5-HTR\(_{2B}\) receptors. The long splice variant of the 5-HTR\(_{3}\) and 5-HTR\(_{4}\) mRNA were found. Expression of the 5-HTR\(_{4}\) mRNA was also detected (Fig. 1B). We found no transcripts for 5-HTR\(_{1A}\), 5-HTR\(_{1D}\), 5-HTR\(_{1F}\), 5-HTR\(_{3C}\), 5-HTR\(_{5}\), and 5-HTR\(_{6}\) receptors in DC (data not shown). Extensive characterization of 5-HTR isotypes present in DC was performed by real-time PCR and relative quantification, at different time points during DC maturation (Fig. 1, C and D). Expression of the transcript of 5-HTR\(_{1B}\), 5-HTR\(_{1E}\), and 5-HTR\(_{2B}\) subtypes significantly decreased, while 5-HTR\(_{4}\) and 5-HTR\(_{6}\) transcripts increased after LPS addition. Expression levels of mRNA for the ligand-gated cation channel 5-HTR\(_{1}\) and the heptahelical 5-HTR\(_{2A}\) subtype did not significantly change during maturation.

Activation of 5-HTR receptors induces intracellular Ca\(^{2+}\) transients

Functional expression of 5-HTR in DC was analyzed by measuring intracellular Ca\(^{2+}\) changes elicited by stimulation of 5-HTR. Stimulation of immature and mature DC with 5-HT induced a rapid and dose-dependent Ca\(^{2+}\) increase both in immature and mature DC although the extent of this response was higher in immature DC (Fig. 2). To study involvement of the different 5-HTR subtypes in Ca\(^{2+}\) transients in immature DC, cells were stimulated with different 5-HTR agonists. Fig. 3A shows that 5-CT which is a preferential agonist at 5-HTR\(_{1}\), 5-HTR\(_{4}\), and 5-HTR\(_{6}\) subtypes, as well as the selective 5-HT\(_{1B}\) agonist AnhC1L (Fig. 3B) induced a spiking Ca\(^{2+}\) rise followed by a slow declining phase. Experiments performed with the 5-HT\(_{1E}\) agonist BRL 54443 (Fig. 3C) and the 5-HT\(_{2}\) agonist DOI (Fig. 3D) show that these subtypes are also functional in immature DC. Moreover, we were able to show that incubation of immature DC with the selective 5-HTR\(_{4}\) antagonist ketanserin (100 μM for 30 min) before stimulation with the 5-HT\(_{2}\) agonist DOI completely abolished the Ca\(^{2+}\) increase induced by this compound, while it failed to block 5-HTR\(_{1}\)- and 5-HTR\(_{3}\)-
mediated responses (data not shown). In contrast to immature DC, mature DC did not respond to any of the above mentioned agonists (Fig. 3E). Unresponsiveness was not due to a generalized defect in the Ca\(^{2+}\) response as the chemokine MIP-3\(\beta\)/CCL19 was able to trigger a Ca\(^{2+}\) response in mature DC (data not shown).

5-HTR\(_3\) is a ligand-gated cation channel triggering Ca\(^{2+}\) influx from the extracellular milieu and consequently plasma membrane depolarization. As shown in Fig. 4, the 5-HTR\(_3\) agonist 2-methyl-5HT induced Ca\(^{2+}\) transients in immature as well as in mature DC. In contrast, the 5-HTR\(_4\) agonist 2-MHT and the 5-HTR\(_7\) agonist 8-HDPAT did not induce any Ca\(^{2+}\) response in immature and mature DC (data not shown). Besides Ca\(^{2+}\) influx through the plasma membrane, Ca\(^{2+}\) transients can be due to mobilization of the ion from the intracellular stores. To better discriminate between the two pathways, DC were stimulated with 5-HT in the absence of extracellular Ca\(^{2+}\). The Ca\(^{2+}\) chelator EGTA reduced 5-HT-induced Ca\(^{2+}\) transients by \(\sim\)20% in immature DC. In contrast, EGTA completely abolished the Ca\(^{2+}\) response in mature DC showing that it was entirely due to influx through the plasma membrane. Chelation of extracellular Ca\(^{2+}\) did not affect the 5-HTR\(_1\) - and 5-HTR\(_2\) -mediated intracellular Ca\(^{2+}\) mobilization induced by AnHCL, BRL 54443, DOI, and 5-CT. In contrast, the 5-HTR\(_3\) -mediated response in immature and mature DC was totally blocked by EGTA (Table I).

Mobilization of Ca\(^{2+}\) from intracellular stores by heptahelical receptors is often mediated via PTX-sensitive G\(_i/o\) proteins (16–18). To study participation of G\(_i/o\)-proteins in 5-HTR\(_1\)-mediated signaling, immature DC were preincubated with PTX and then stimulated with 5-CT, AnHCL, and BRL 54443 (Fig. 5). PTX almost completely abolished responses induced by these agonists, showing that its inhibitory effects were not due to cytotoxicity (data not shown).

Table I. Effect of extracellular Ca\(^{2+}\) chelation on intracellular Ca\(^{2+}\) transients induced by different 5-HT receptor agonists in immature and mature DC

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<th>5-HT</th>
<th>5-CT</th>
<th>AnHCL</th>
<th>BRL 54443</th>
<th>DOI</th>
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<tr>
<td>Immature DC Medium</td>
<td>0.89 ± 0.04</td>
<td>1.14 ± 0.04</td>
<td>1.34 ± 0.06</td>
<td>1.24 ± 0.05</td>
<td>1.19 ± 0.05</td>
<td>1.20 ± 0.03</td>
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<tr>
<td>Mature DC Medium</td>
<td>0.84 ± 0.03</td>
<td>1.03 ± 0.04</td>
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<td>0.86 ± 0.05</td>
<td>1.00 ± 0.04</td>
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</tbody>
</table>

In immature and mature DC were loaded with fura-2/AM as reported in Materials and Methods and stimulated with 10\(^{-5}\) M of the indicated agonists, in the absence or presence of 4 mM EGTA. Results are expressed as means ± SEM (n = 5).
5-HT increases cAMP levels and stimulates cytokine production in mature DC

**FIGURE 7.** Stimulation of serotoninergic receptors induces secretion of IL-8 from mature but not immature DC. Immature and mature DC were stimulated with the indicated concentrations of 5-HT (A). Supernatants were collected 24 h after stimulation and IL-8 concentration was measured by ELISA. Results are given as mean ± SEM (n = 4). Mature DC were stimulated with the indicated concentrations of different 5-HTR agonists (B). Data are presented as mean ± SEM (n = 4). Time dependency of the 5-HTR induced IL-8 production in mature DC (C). DC were stimulated with optimal concentrations of the indicated agonists. Stimulation of cells and IL-8 measurement were performed as described in A. Data are presented as mean ± SEM (n = 3).

**FIGURE 8.** 5-HTR stimulation induces IL-1β production in mature but not immature DC. Immature and mature DC were stimulated with the indicated concentrations of 5-HT. Supernatants were collected 24 h after stimulation and IL-1β content was measured by ELISA (A). Results are given as mean ± SEM (n = 4). Mature DC were stimulated with the indicated concentrations of 5-HTR agonists (B). Stimulation of cells and IL-1β quantification were as for A. Results are given as mean ± SEM (n = 4). Time dependency of the 5-HTR induced IL-1β production in mature DC (C). DC were stimulated with optimal concentrations of the indicated agonists. Stimulation of cells and IL-1β measurement were performed as described in A. Data are presented as mean ± SEM (n = 3).

5-HT increases cAMP levels and stimulates cytokine production in mature DC

5-HTR₄ and 5-HTR₇ couple to Gₛ proteins and stimulate adenylyl cyclase. To investigate functional coupling of 5-HTR₄ and 5-HTR₇ in DC, cAMP levels were analyzed after stimulation with 5-HT.

(D). Total RNA was isolated from DC (1 x 10⁶) stimulated with LPS 3 μg/ml in the absence or presence of 10⁻⁴ M 5-HT for 4, 12, and 24 h. IL-8 mRNA expression was quantified as described in Materials and Methods. D. Number of transcripts is normalized to the number of copies of GAPDH ones. Data are means ± SEM (n = 4).
the 5-HTR₄ agonist 2-MHT as well as the 5-HTR₇ agonist 8-HDPAT. 5-HT, 2-MHT, and 8-HDPAT did not elicit cAMP increase in immature DC, whereas they induced accumulation of this second messenger in a concentration-dependent manner in LPS-matured DC (Fig. 6).

Recent evidence suggests that 5-HT modulates the production of cytokines in T cells and monocytes (3, 4). Fig. 7A shows that 5-HT added together with LPS, dose-dependently increased the production of IL-8 in immature DC, whereas it did not affect cytokine production in immature DC, whereas they induced accumulation of this second messenger in a concentration-dependent manner in LPS-matured DC (Fig. 6).

To study involvement of different 5-HT receptor subtypes on IL-8 and IL-12 release, experiments with the selective 5-HT receptor antagonist RS-39604 and the 5-HT receptor antagonists pimozide or SB-269970. DC were then stimulated with 10⁻⁴ M 2-MHT, 8-HDPAT, or 10⁻³ M 2-Me5HT. IL-8 and IL-1β production were measured after 24 h. Data are given in picograms per milliliter × 200,000 cells and represent means ± SEM (n = 3).

![Image](86x95 to 260x354)

**FIGURE 9.** 5-HTR inhibits the production of TNF-α and IL-12 in mature DC. Immature and mature DC were stimulated with the indicated 5-HT concentrations. Supernatants were collected 24 h after stimulation; TNF-α (A) and IL-12 (B) were measured by ELISA. Results are given as mean ± SEM (n = 4).

### Table II. Effect of the 5-HTR₄ antagonist RS-39604 and the 5-HTR₇ antagonists pimozide or SB-269970 on IL-1β and IL-8 production

<table>
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<th>2Me5HT</th>
<th>2-MHT</th>
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<tr>
<td>IL-8 Production</td>
<td>35,000 ± 5,000</td>
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<td>IL-1β production</td>
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<td>3,100 ± 350</td>
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<td>Pimozide</td>
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<tr>
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<td>SB-269970</td>
<td>Pimozide</td>
<td>SB-269970</td>
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</tbody>
</table>

* LPS-maturing DC were preincubated with 10⁻⁷ M of the selective 5-HTR₄ antagonist RS-39604 or the 5-HTR₇ antagonists pimozide or SB-269970. DC were then stimulated with 10⁻⁴ M 2-MHT, 8-HDPAT, or 10⁻³ M 2-Me5HT. IL-8 and IL-1β production were measured after 24 h. Data are given in picograms per milliliter × 200,000 cells and represent means ± SEM (n = 3).

![Image](DOI 7230)

**Table III. Effect of the selective 5-HTR subtype agonists on TNF-α and IL-12 production**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2Me5HT</th>
<th>2-MHT</th>
<th>8-HDPAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12 Production</td>
<td>7890 ± 570</td>
<td>8940 ± 680</td>
<td>8650 ± 590</td>
<td>8650 ± 590</td>
</tr>
<tr>
<td>TNF-α Production</td>
<td>8290 ± 640</td>
<td>9100 ± 745</td>
<td>8300 ± 820</td>
<td>8300 ± 820</td>
</tr>
<tr>
<td>Control</td>
<td>AnHCL</td>
<td>BRL 54443</td>
<td>DOI</td>
<td>2-Me5HT</td>
</tr>
<tr>
<td>IL-12</td>
<td>7567 ± 560</td>
<td>7230 ± 920</td>
<td>8100 ± 670</td>
<td>2750 ± 490</td>
</tr>
<tr>
<td>TNF-α</td>
<td>8290 ± 640</td>
<td>9100 ± 745</td>
<td>8650 ± 590</td>
<td>8650 ± 590</td>
</tr>
<tr>
<td>Control</td>
<td>2-MHT</td>
<td>8-HDPAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>2750 ± 490</td>
<td>2500 ± 580</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>4100 ± 790</td>
<td>3500 ± 570</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* LPS-maturing DC were stimulated with 10⁻⁴ M of the indicated 5-HT-receptor agonists for 24 h. IL-12 and TNF-α production were measured as reported in Materials and Methods. Data are given as means ± SD (n = 3). Global differences between groups: p ≤ 0.0001 (ANOVA).

* p ≤ 0.01

* p ≤ 0.05 compared with untreated cells (Tukey’s multiple comparison test).
Discussion

5-HT is present in the periphery at high concentrations, in platelets, basophils, and mast cells (2) and it is released during platelet aggregation or IgE stimulation. There is accumulating evidence to support a regulatory function of 5-HT in the immune system (2–6). A role for 5-HT in the pathogenesis of bronchial asthma has also been recently proposed (8). Pharmacological and molecular studies revealed the existence of different 5-HTR subtypes classified either as ligand-gated cation channels or in the G protein-coupled receptor superfamily.

In this study, we show that DC expressed several functional 5-HTR subtypes. In addition, we also found that 5-HTR mRNA expression levels were modulated during DC maturation. Immature, compared with mature, DC expressed higher mRNA levels of the 5-HTR1B, 5-HTR1E, and 5-HTR2B subtypes. Comparable mRNA levels of the two splice variants of 5-HTR4 and 5-HTR7A were found in immature and mature DC, whereas mRNA levels of the 5-HTR4 and 5-HTR7 subtypes were higher in mature DC.

To investigate functional expression of the different 5-HTR subtypes during DC maturation, we analyzed in more detail the intracellular signaling pathways activated by 5-HT. 5-HTR1B, 5-HTR1E, and 5-HTR2A receptors couple to PTX-sensitive Gαi, as well as to PTX-insensitive Gαq proteins. Stimulation of these receptors also activates phospholipase C which cleaves phosphoinositides into diacylglycerol and inositol 1,4,5-trisphosphate, inducing mobilization of Ca2+ from the intracellular stores. By monitoring agonist-dependent Ca2+ changes, we showed that 5-HTR1B, 5-HTR1E, and 5-HTR2A receptors were functional and coupled to Gαi proteins only in immature DC. Moreover, we found that the cation channel 5-HTR1E was functional both in immature and mature DC. These findings suggest that while in immature DC, 5-HT induced intracellular Ca2+ concentration changes via 5-HTR1E and 5-HTR2A-mediated Ca2+ mobilization from the intracellular stores, besides the ligand-gated cation channel 5-HTR1E-mediated Ca2+ influx. In mature DC, the only active pathway seemed to be that mediated by 5-HTR2A. 5-HTR1E, and 5-HTR2A coupled via Gαi to stimulate adenylyl cyclase (22, 27). Functional expression of these two receptors was demonstrated in mature DC. We showed that 5-HT induced an increase in cAMP concentration in these cells. The shift in 5-HT-induced Gαi protein-dependent Ca2+ response to adenylyl cyclase-mediated cAMP formation during the maturation process was well in accordance with the increased mRNA expression levels of the 5-HTR4 and 5-HTR7 subtypes during DC maturation. However, to explain this functional shift one cannot exclude other mechanisms besides transcriptional down- and/or up-regulation of single 5-HTR subtypes. Retention of receptor molecules into submembraneous vesicles or posttranslational modifications of G protein subunits can also be hypothesized.

To get insight into the physiological significance of 5-HT in DC, cytokine secretion was analyzed. We found that stimulation of 5-HTR1A, 5-HTR2A, and 5-HTR4 subtypes mediated the release of IL-1β and IL-8. However, mRNA analyses suggested that 5-HT modulated secretion of IL-1β and IL-8 by two different mechanisms. Enhanced IL-8 mRNA levels upon stimulation of DC with 5-HT would suggest a transcriptionally regulated effect. In contrast, unchanged mRNA levels of IL-1β in immature and mature DC indicate that 5-HT would affect a posttranscriptional regulatory step in IL-1β production. In this context, it might be of interest that 5-HT has been recently involved in the pathogenesis of asthma (8). Several studies have shown that allergen challenge causes, in humans as well as in animal models, an IL-8-mediated recruitment of neutrophils in the lung, and also an IL-1β-dependent alteration of airway smooth muscle responses (38, 39). Therefore, it can be hypothesized that in patients with acute severe asthma, IL-8 released by 5-HT-activated DC may cause neutrophil infiltration, and that secretion of IL-1β would then exacerbate the proinflammatory changes due to airway smooth muscle hyperresponsiveness. DC are critical effectors in both initiating and modulating immune responses because they capture, process, and transport Ags to secondary lymphoid organs, where they prime T cells (30, 31). Depending on the microenvironment, DC can regulate the outgrowth of T cell subsets. In the presence of IL-12 they induce Th1 cells, whereas with IL-4 there is induction of Th2 cell subsets (37, 40).

In summary, our study shows that 5-HT activates, in a maturation-dependent manner, different DC signaling pathways. These data further stress the immunomodulatory role of 5-HT at peripheral sites.

References

receptor: molecular cloning and pharmacological characterization of two splice variants. \textit{EMBO J.} 14:2806.


