

Downregulation of basophil-derived IL-4 and *in vivo* T_H2 IgE responses by serotonin and other organic cation transporter 3 ligands

Elke Schneider, PhD,^a François Machavoine, MSc,^a Rachel Bricard-Rignault, MSc,^a Mélanie Levasseur, MSc,^a Anne France Petit-Bertron, PhD,^a Sophie Gautron, PhD,^b Jean-Antoine Ribeil, MD, PhD,^a Jean-Marie Launay, MD, PhD,^c Salah Mecheri, PhD,^d Francine Côté, PhD,^a and Michel Dy, PhD^a Paris, France

Background: Murine basophils can contribute to the T_H2 polarization of the immune response by providing rapidly large amounts of IL-4, which suggests that pharmacologic downregulation of this cytokine might provide a strategy to attenuate pathologies associated with excessive production. **Objective:** We examined a number of physiological and pharmacologic ligands of the organic cation transporter 3 (OCT3), a membrane carrier of biogenic amines, for their inhibitory effect on IL-4 production by basophils, selecting the most efficient compounds for *in vivo* evaluation in basophil-dependent experimental models.

Methods: IL-4 production by basophils isolated *ex vivo* or from bone marrow cultures was assessed in response to various stimuli with or without biogenic monoamines or pharmacologic analogs. Selected compounds were administered *in vivo* to examine their effect on levels of circulating IgE generated during a basophil-dependent T_H2 response and on basophil activation in mice receiving IL-33.

Results: We found a drastic decrease in IL-4 production by stimulated basophils on exposure to serotonin (5-hydroxytryptamine [5-HT]) that is taken up by basophils through the specific high-affinity transporters serotonin transporter and the polyspecific, high-capacity organic cation transporter 3 (OCT3; or Slc22a3) but inhibits their function exclusively through the latter. This downregulation is likewise observed *in vivo* in response to 5-HT and other OCT3 ligands, as well as in human basophils sorted from PMBCs of nonatopic donors.

Conclusions: We provide evidence for a new means of downregulating IL-4 production by basophils, both *in vitro* and

in vivo, through OCT3 targeted by 5-HT and pharmacologic ligands. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Basophils, IL-4, T_H2-type immune response, organic cation transporter 3

The contribution of basophils to the orientation of the immune response in murine models is widely acknowledged.¹⁻⁴ We provided the first evidence for a basophil population with low granular content in murine bone marrow using *in situ* hybridization with a riboprobe encoding histidine decarboxylase (HDC; the histamine-forming enzyme) and ultrastructural criteria.^{5,6} These cells constitute an excellent source of pro-T_H2 cytokines and histamine in response to growth factors, such as IL-3, and other stimuli^{7,8} and are recruited to peripheral organs, where they exert beneficial or deleterious functions in pathologic situations, such as nematode infection or allergic responses, respectively.⁹⁻¹²

Basophils have been detected in the lungs of patients with allergic asthma, in which their incidence increases in fatal cases.¹³ The critical role of basophils in the development of chronic allergic inflammation has been established recently in a murine model by using transgenic mice that generate 2,4,6-trinitrophenol-specific IgE.⁴ This study took advantage of new procedures of *in vivo* depletion and adoptive transfer to demonstrate that basophils are indispensable for the late-phase allergic reaction.

Similar approaches have confirmed the nonredundant role of basophils in orchestrating the immune response because of their capacity to generate IL-4 rapidly more efficiently than other producer cells on exposure to an increasing number of stimuli.^{1,14,15} It has been established that basophils can be targeted in an IgE-independent manner by allergen proteases, such as papain, which promotes their recruitment and stimulation in draining lymph nodes, initiating a T_H2 immune response.¹⁶ More recently, their immunoregulatory potential has been further extended to acquired immunity against ticks and their expression of MHC class II molecules.¹⁷⁻²¹

These data prompted us to investigate whether potentially harmful activities of basophils could be attenuated by downregulating their pro-T_H2 cytokine profile. We have previously reported that histamine exerts a negative control of its own synthesis and that of associated cytokines on uptake by basophils through the bidirectional organic cation transporter 3 (OCT3).²² Here we extend this study in search of more efficient compounds to

From ^aUniversité Paris Descartes, Faculté de Médecine, CNRS UMR 8147 "Cytokines, Hematopoiesis and Immune Response," Hôpital Necker; ^bINSERM U952, CNRS UMR 7224, UPMC Univ Paris 06; ^cService de Biochimie et Biologie Moléculaire/Equipe Associée (EA) 3621, Hôpital Lariboisière; and ^dUnité BIHP-Bt Sergent, Institut Pasteur.

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Reprint requests: Elke Schneider, PhD, CNRS UMR 8147, Hôpital Necker, 161 rue de Sèvres, 75015 Paris, France. E-mail: elke.schneider@parisdescartes.fr.
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Abbreviations used

5-HT:	5-Hydroxytryptamine
BMC:	Bone marrow cell
BMDb:	Bone marrow–derived basophil
CYP450:	Cytochrome P450
Dat:	Dopamine transporter
DNP:	Dinitrophenyl
<i>Gapdh</i> :	Glyceraldehyde-3-phosphate dehydrogenase
HDC:	Histidine decarboxylase
Net:	Norepinephrin transporter
OCT3:	Organic cation transporter 3
SERT:	Serotonin transporter

downregulate basophil activities both *in vitro* and *in vivo*. Among the monoamines tested, serotonin, or 5-hydroxytryptamine (5-HT), turned out to be the most potent inhibitor, suppressing basophil-derived IL-4 production completely. This effect was relevant *in vivo* in 2 experimental settings, namely treatment with IL-33, which primes basophils for increased IL-4 production,²³ and immunization with papain, which induces a strong basophil-dependent T_H2 bias.¹⁸ Both responses were strongly inhibited by 5-HT, which is internalized through OCT3, as well as by ciproxifan and clobenpropit, which block this bidirectional membrane carrier. Finally, IL-4 and IL-13 production in response to 5-HT was likewise inhibited in stimulated human basophils isolated from peripheral blood of nonatopic donors.

METHODS**Mice and reagents**

Six to 8-week-old specific pathogen–free, female C57BL/6J mice were purchased from CERJ (Les Genest St Isle, France). *Hdc*^{−/−} mice were generated by Ohtsu et al.²⁴ *Sert*^{−/−} and *Oct3*^{−/−} mice bred on a C57BL/6J genetic background were originally developed by Bengel et al.²⁵ and Zwart et al.,²⁶ respectively. Animal experiments were performed according to the guidelines of the French Institutional Committee.

Recombinant preparations of IL-3 (murine and human), murine IL-33, murine GM-CSF, and murine and human DuoSet ELISA kits for IL-4, IL-6, and IL-13 were purchased from R&D Systems (Abington, United Kingdom). Clobenpropit dihydrobromide was from Tocris (Ellisville, Mo). All other OCT3 and serotonin transporter (SERT) ligands, as well as serotonin hydrochloride, calcium ionophore (A23187), papain, and IgE (SPE-7), were from Sigma-Aldrich (St Louis, Mo). Tritiated serotonin dihydrochloride and tritiated histamine dihydrochloride were provided by PerkinElmer (Waltham, Mass). Anti-human and anti-mouse FcεRIα mAbs (clone AER-37 [CRA1] and clone MAR-1, respectively) were purchased from eBioscience (San Diego, Calif), whereas anti-dinitrophenyl (anti-DNP) ascite and DNP were provided by S. Mecheri.

Cell cultures, flow cytometry, and sorting

Bone marrow cells (BMCs) were prepared as reported before⁵ and adjusted to a final concentration of 2.5×10^6 /mL in culture medium (minimal essential medium) supplemented with 10% horse serum (all from Gibco, Grand Island, NY). Various doses (10^{-5} to 10^{-9} mol/L) of the drugs were added shortly before IL-3 (10 ng/mL), GM-CSF (10 ng/mL), A23187 (10^{-6} mol/L), anti-FcεRIα (1 μg/mL), and DNP (0.1 μg/mL) plus anti-DNP ascite (1:5000), followed by a 24-hour incubation at 37°C and 5% CO₂. In some experiments BMCs were enriched for basophils by depleting NK1.1⁺ cells, followed by positive selection of the CD49b⁺ subset with the RoboSep automaton (StemCell Technologies, Vancouver, British Columbia, Canada). This procedure yielded a 50- to 100-fold enriched fraction in terms of IL-3–induced IL-4 production that comprised approximately 30% FcεRIα⁺c-kit[−] cells. Basophil-enriched populations were also generated from total BMCs cultured for 8

days with IL-3, as described previously.²² Basophils were identified by their FcεRIα⁺c-kit[−] or CD49b⁺c-kit[−] phenotype. In some experiments these basophil-enriched populations were further sorted either electronically with a FACS Vantage (Becton Dickinson, Franklin Lakes, NJ) cell sorter or magnetically with a RoboSep automated pan-NK positive selection kit (StemCell Technologies). Sorted cells had 98% or greater purity on reanalysis and displayed the morphology reported for murine basophils after May-Grünwald-Giemsa staining. The following appropriately labeled antibodies were used: anti-mouse CD117 (2B8), CD11b (M1/70), CD49b (Hma2), NK1.1 (PK136), and isotype controls (all from PharMingen, San Diego, Calif). Anti-FcεRIα (MAR-1) was from eBioscience. Cell viability was assessed among gated basophils after annexin-V/7-aminocoumarin D staining by using a commercial kit from Becton Dickinson.

Peripheral blood cells were recovered after therapeutic phlebotomy in the hematology center of the Necker Hospital with the patients' consent and according to the guidelines of the local ethics committee. Basophils were purified by using the human basophil enrichment kit from StemCell Technologies, according to the manufacturer's instructions. Sorted unstimulated basophils had 95% or greater purity on reanalysis. Purified basophils were incubated in Iscove modified Dulbecco medium supplemented with 10% horse serum and stimulated with IL-3 (10 ng/mL) plus A23187 (10^{-6} mol/L) or IL-3 plus anti-human FcεRIα mAb (1 μg/mL) with or without 5×10^{-4} mol/L 5-HT.

***In vivo* treatment with IL-33, papain, monoamines, and OCT3 ligands**

Mice received a daily intraperitoneal injection of 1 μg of mrIL-33 for 4 days with or without histamine (10 mg/kg), 5-HT (10 mg/kg), or ciproxifan (5 mg/kg) administered together in 200 μL of saline. For T_H2 induction, animals were immunized subcutaneously in the rear footpads with 50 μL of saline in which 50 μg of papain with or without monoamines and related compounds was dissolved. Histamine and 5-HT were administered intraperitoneally at a concentration of 10 mg/kg 4 hours before immunization and twice daily for 3 days after. Clobenpropit was injected with the same schedule and route of administration at a concentration of 20 mg/kg. Control mice received injections of saline. BMCs from mice treated with OCT3 ligands or saline were recovered on day 4 after IL-33 administration. Total IgE levels were measured on days 14 and 21 after immunization with papain with or without OCT3 ligands by using an ELISA kit from Becton Dickinson.

Assessment of cytokine production and radiolabeled serotonin and histamine uptake

IL-4, IL-6, and IL-13 levels were measured by means of ELISA in cell supernatants after a 24-hour incubation, and histamine was quantified by using an automated continuous-flow spectrofluorometric technique. For 5-HT uptake experiments, 2×10^5 total BMCs, 2.5×10^4 basophil-enriched natural killer cell–depleted CD49b⁺ cells, and 5000 electronically sorted FcεRIα⁺c-kit[−] cells were distributed into round-bottom 96-well polypropylene plates (Costar, Cambridge, Mass). Unless stated otherwise, the cells were incubated (37°C and 5% CO₂) for 3 hours with 3 mCi/mL tritiated serotonin dihydrochloride (10^{-7} mol/L; 20.5 Ci/mmol) and tritiated histamine dihydrochloride (1.6×10^{-7} mol/L; 15.5 Ci/mmol) in a final volume of 100 μL. Competition assays were carried out as previously described.²²

mRNA expression

RNAs were extracted from sorted bone marrow–derived basophils by using the RNeasy microkit (Qiagen, Hilden, Germany), according to the supplier's recommendations. Primers and probes for murine *Il4*, *Il6*, *Hdc*, and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) real-time PCR were designed by using the Computer Primer Express software (Applied Biosystems, Les Ulis, France). All oligonucleotides were purchased as HPLC-purified molecules from Eurogentec (Angers, France). PCR reactions contained 1 μL of cDNA samples, 100 nmol/L of each primer, 200 nmol/L of the specific probe, and 60 nmol/L passive reference (Rox) in 24 μL of Taqman Universal

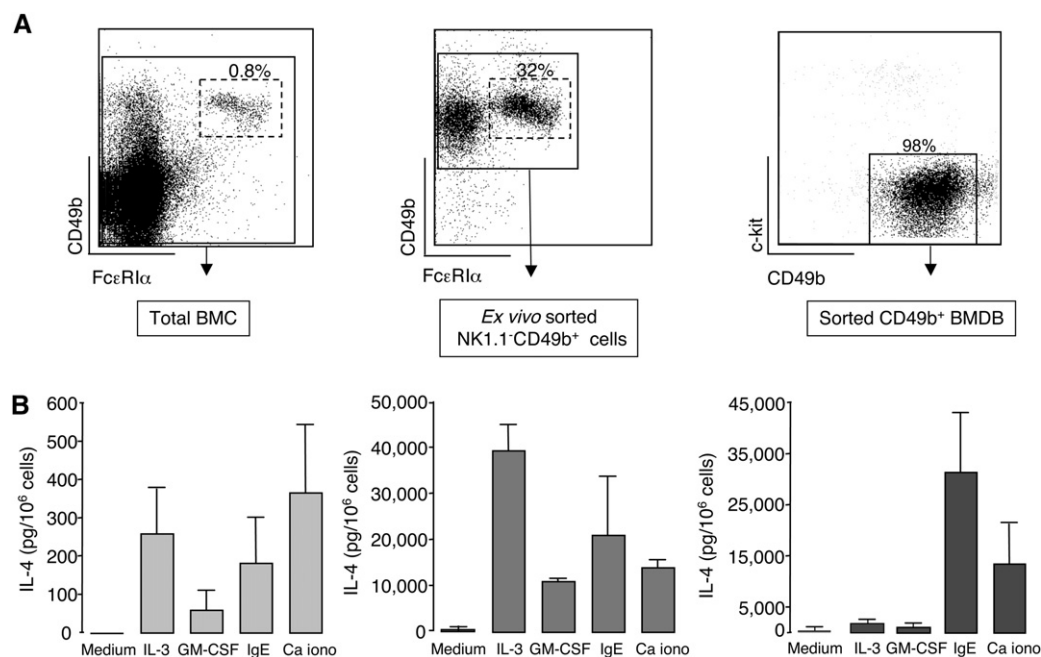


FIG 1. Basophils are proficient IL-4 producers in response to several stimuli. **A**, Sorting of basophils isolated *ex vivo* from BMCs or derived from culture. **B**, IL-4 production by bone marrow populations containing different proportions of basophils. IL-4 was measured in supernatants after a 24-hour incubation in the presence of the stimuli indicated ($n = 4$). *Ca iono*, Calcium ionophore.

MasterMix (Applied Biosystems). Each amplification was performed in triplicate by using the following conditions: 2 minutes at 50°C and 10 minutes at 94°C, followed by 40 cycles of 15 seconds at 94°C and 30 seconds at 60°C. All data were normalized to an internal standard, *Gapdh* expression in each sample, and expressed as relative expression by using the DDC_T method, as described in user bulletin no. 2 from Applied Biosystems.

The probes carried a 5' FAM reporter label and a 3' dark quencher group and were synthesized by Eurogentec (Liège, Belgium).

Oligonucleotides for real-time PCR were as follows: *Gapdh* forward, ATG TGT CCG TCG TGG ATC TGA; *Gapdh* reverse, GAT GCC TGC TTC ACC ACC TT; *Gapdh* probe: CCT GGA GAA ACC TGC CAA GTA TGA TGA CAT; *Il4* forward, TCA TCG GCA TTT TGA ACG AG; *Il4* reverse, TTG GCA CAT CCA TCT CCG T; *Il4* probe, TCA CAG GAG AGG GGA CGC CAT GC. Oligonucleotides for qualitative PCR analyses were as follows: β_2 -microglobulin (*B2m*) forward, TGA CCG GCT TGT ATG CTA TC; *B2m* reverse, CAG TGT GAG CCA GGA TAT AG; *Oct3* forward, GGA AGC AAG GAG AGA AAG CC; *Oct3* reverse, CCA CAG ACA GAC GCC AGG AT; *Sert* forward, GCT TCG TCT CTG GCT TTG TCA T; *Sert* reverse: GCT ATC CAA ACC CAG CGT GA; norepinephrin transporter (*Net*) forward, GCT GCT CTG TCT GAT GGT CG; *Net* reverse, TCC TTG AGG CGG TAG AAG TCA; dopamine transporter (*Dat*) forward, GCT ACA TCG GCATCG AGA GC; *Dat* reverse, CCA GTA CAG GTT GGG TCG CT.

Statistics

Data are expressed as means $\pm$ SEMs, and the standard Student 2-tailed *t* test was used to establish statistical significance.

RESULTS

Comparison between freshly isolated and bone marrow-derived basophils for IL-4 production

Most functional studies of murine basophils have been performed with BMCs cultured for 8 to 10 days with IL-3, yielding 10- to 30-fold enriched populations. This procedure

raises the question of the physiological relevance of data obtained with "primed" cells relative to unstimulated basophils sorted *ex vivo*. We addressed this issue, comparing these 2 populations for their response to various stimuli according to the phenotypic criteria established previously.⁴ Depletion of NK1.1⁺ cells from freshly isolated bone marrow followed by positive magnetic selection of the CD49b⁺ fraction yielded a population comprising 30% to 40% FcεRIα⁺c-kit⁺ basophils (Fig 1, A). IL-4 secretion was increased accordingly whether the cells were stimulated with A23187, IgE (clone SPE7), GM-CSF, or IL-3 (Fig 1, B). Electronic sorting of the FcεRIα⁺c-kit⁺ population further increased IL-3-induced IL-4 production, reaching 75 ng/10⁶ cells in a typical experiment. The CD49b⁺FcεRIα⁺ population produced no detectable amounts of IL-4. These results imply that basophils are the main IL-4 producers, which was further corroborated by the fact that the NK1.1⁺CD49b⁺ population responded to anti-FcεRIα mAb and DNP/anti-DNP mAb (9696 and 9372 pg of IL-4 per 10⁶ cells, respectively, as assessed in a single experiment).

Compared with its *ex vivo* counterpart, the CD49b⁺ population derived from BMC cultures (bone marrow-derived basophils [BMDBs]; Fig 1, B) was much less responsive to IL-3 and GM-CSF in terms of IL-4 production conversely to IgE or Ca ionophore, even though it comprised 98% or greater FcεRIα⁺c-kit⁺ basophils (Fig 1, A). This relative unresponsiveness suggests that the long-term exposure to the growth factor has set off a negative feedback control, possibly mediated through the suppressive signal transducer and activator of transcription-dependent suppressors of cytokine signaling pathway²⁷ or other suppressive pathways. For this reason, culture-derived basophils cannot adequately replace their freshly isolated equivalent to evaluate inherent functions that are more accurately assessed by using unstimulated whole or basophil-enriched BMCs.

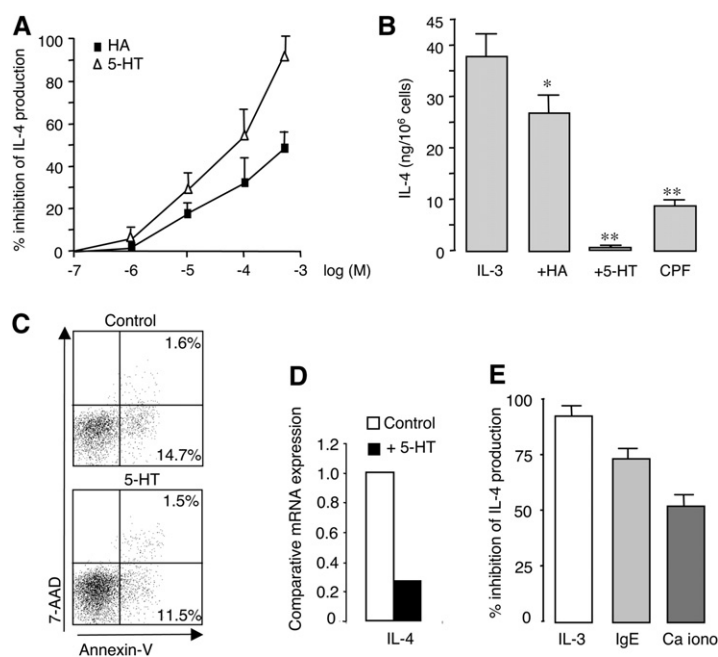


FIG 2. 5-HT downregulates IL-4 production by basophils on protein and transcription levels. **A**, Dose-response curve ($n = 4$). HA, Histamine. **B**, 5-HT, histamine, and ciprofloxacin (CPF; all at 5×10^{-4} mol/L) inhibit IL-3-induced IL-4 production by basophils sorted *ex vivo* ($n = 3$). * $P \leq .05$. ** $P \leq .001$. **C**, 5-HT does not impair basophil viability. 7-AAD, 7-Aminoactinomycin D. **D**, 5-HT inhibits IL-4 mRNA expression. **E**, 5-HT inhibits IL-4 production in response to several stimuli ($n = 3$). Ca iono, Calcium ionophore.

Inhibition of IL-4 production by stimulated murine basophils exposed to serotonin

Few negative regulators of basophils have been reported thus far, excepting IFN- α , which inhibits IL-3 priming of human basophil cytokine secretion,²⁸ and histamine itself, which decreases its own synthesis and that of associated cytokines once it reaches critical intracellular levels.²² Having established that classical immunosuppressive cytokines, such as IL-10, IL-1, IFN- γ , or TGF- β , had no effect on IL-4 production by stimulated basophils (data not shown), we investigated whether biogenic amines other than histamine exerted a similar or stronger inhibition. Among the numerous molecules tested in this study (data not shown), 5-HT turned out to be the most potent inhibitor, as shown in the dose-response curve in Fig 2, A. This monoamine reduced IL-4 production by IL-3-induced total BMCs more drastically than histamine itself, approaching 100% inhibition at a concentration of 5×10^{-4} mol/L. Other monoamines of the neuroimmunologic axis, such as dopamine or noradrenaline, had a less consistent effect (data not shown). IL-3-induced production of IL-6 and histamine was likewise inhibited by 5-HT (see Fig E1 in this article's Online Repository at www.jacionline.org), which had a similar effect on *ex vivo*-sorted basophil-enriched NK1.1⁺CD49b⁺ cells stimulated with IL-3 that were less affected by histamine or the OCT3 ligand ciprofloxan (Fig 2, B). 5-HT did not impair cell viability among gated basophils after annexin-V/7-aminoactinomycin D staining (Fig 2, C) but diminished IL-4 levels by inhibiting IL-3-induced expression of the encoding mRNA in purified bone marrow-derived CD49b⁺c-kit⁺ basophils analyzed by means of real-time PCR (Fig 2, D). The inhibition of IL-4 production occurred also in response to other basophil activators, such as IgE and A23187 (Fig 2, E).

Involvement of the membrane transporter OCT3 in 5-HT-induced downregulation of basophil functions

The high concentrations of 5-HT required for downregulation of basophil functions argued against the implication of specific receptor activation, prompting us to examine the expression of monoamine transporters in purified BMDBs. As shown in Fig 3, A, transcripts encoding the specific 5-HT transporter SERT, as well as the polyspecific cation transporter OCT3, were expressed (Fig 3, A), conversely to *Dat* and *Net*, the specific transporters of dopamine and noradrenaline, respectively, which were only detected in total BMCs.

Because basophils are most frequent in the bone marrow, we evaluated 5-HT uptake by exposing total cells to the radiolabeled monoamine. As shown in Fig 3, B, a substantial amount of tritiated 5-HT was internalized at 37°C, reaching a maximum within 3 hours, whereas cells incubated at 4°C did not retain any label in these conditions, suggesting uptake by specific carriers rather than binding to 5-HT receptors. In support of this notion, fluoxetine and citalopram, 2 specific inhibitors of SERT, did decrease labeling with tritiated 5-HT ($79.3\% \pm 3.1\%$ and $79.0\% \pm 3.05\%$ reduction in response to fluoxetine and citalopram, respectively, at a concentration of 10^{-6} mol/L; means $\pm$ SEMs from 3 experiments).

To prove that basophils (<1% of BMCs, as shown in Fig 1, A) are the major subset involved in the uptake of 5-HT, we repeated the experiment with purified BMDBs that retained around 200 times more radiolabeled compound than total BMCs ($119,974$ vs 645 fmol/ 10^6 cells in a typical experiment). Labeling was more than 90% reduced by the SERT inhibitor fluoxetine, as well as in basophils from *Sert*^{-/-} mice, which proved that at the low concentrations of the radioactive compound ($1.6 \times$

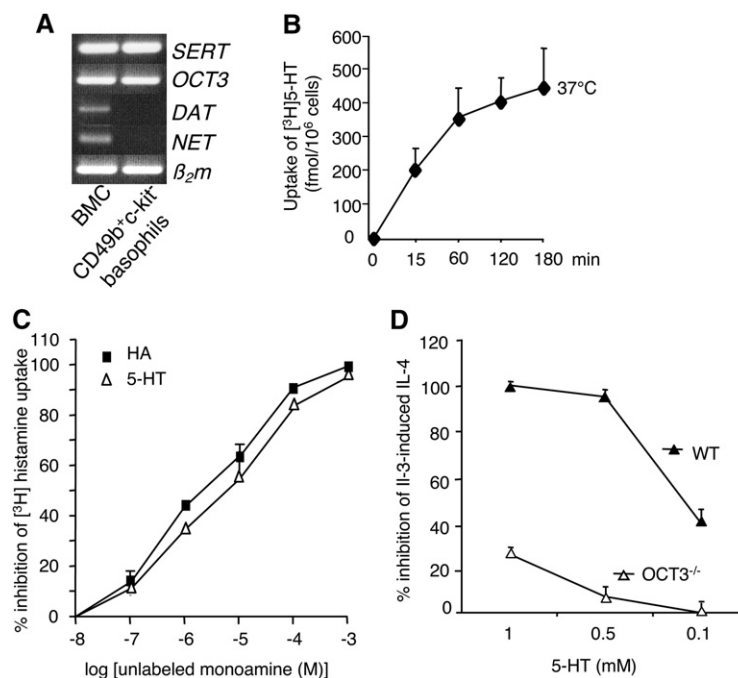


FIG 3. SERT mediates the high-affinity uptake of 5-HT by murine basophils but not its inhibitory effect on IL-4 production. **A**, Concomitant OCT3 and SERT expression in purified bone marrow-derived CD49b⁺c-kit⁻ basophils. β_2m , β_2 -Microglobulin. **B**, Kinetics of 5-HT uptake ($n = 4$). **C**, Displacement of tritiated histamine taken up by BMCs by unlabeled monoamines ($n = 3$). HA, Histamine. **D**, 5-HT-induced inhibition of basophil activities is abolished in *Oct3*^{-/-} mice ($n = 3$). WT, Wild-type.

10^{-7} mol/L), 5-HT is mainly internalized through its high affinity transporter.

By contrast, as shown in Table I, downregulation of IL-4 production at high concentrations of 5-HT persisted in the presence of the specific SERT inhibitors fluoxetine and citalopram. Similarly, BMCs recovered from *Sert*^{-/-} mice remained susceptible to inhibition, even though the high-affinity uptake of tritiated 5-HT was abolished, which ruled out a major involvement of the 5-HT-specific transporter SERT in the downregulation of IL-4 production.

We have reported before that basophils express the bidirectional transporter OCT3 (Fig 3, A), which is involved in histamine uptake and release after neosynthesis.²² We found that unlabeled 5-HT competed with tritiated histamine for internalization through OCT3 (Fig 3, C), suggesting that the 2 amines share a common site of interaction. The implication of the high-capacity transporter in the downregulation of IL-4 production by 5-HT was also supported by the lack of effect in *Oct3*^{-/-} mice (Fig 3, D).

Histamine-dependent and independent downregulation of IL-4 production by basophils

We hypothesized that 5-HT might reduce IL-4 production by basophils indirectly by blocking OCT3, as do specific inhibitors or substrates of this transporter.²² Thereby it would cause intracellular accumulation of histamine, which is actively produced by basophils in response to IL-3 and partially secreted through the bidirectional transporter, thus triggering a negative feedback signal. This was not the case because the downregulation persisted in histamine-free *Hdc*^{-/-} mice ($98\% \pm 4.5\%$ of wild-type control

animals in the case of IL-3-induced IL-4 production, $n = 3$). Exposure to 5-HT also did not increase intracellular histamine levels (Table II), and inhibition was maintained in *Hdc*^{-/-}*ITph1*^{-/-} mice, in which histamine and 5-HT are no longer produced, ruling out the contribution of both intracellular histamine and newly synthesized 5-HT (data not shown).

Conversely to 5-HT, other OCT3 ligands, such as clobenpropit and ciproxifan, depended on endogenous histamine because they lost their effect in *Hdc*^{-/-} mice and could increase intracellular levels of histamine, most likely by blocking part of its release through OCT3 (Table II). Other molecules related to histamine and 5-HT, such as histidine, 5-hydroxytryptophan, or melatonin, modified neither uptake nor IL-4 production by basophils, whereas monoamines, such as tyramine, octopamine, tryptamine, and β -phenylethylamine, inhibited uptake without affecting the biological activity. Polyamines and the H₁ receptor antagonist cetirizine had no effect at all (not shown).

Because metabolites of 5-HT, such as H₂O₂ and reactive oxygen species, have been implicated in transcriptional repression,²⁹ we examined their potential role as signal carriers. Neither monoamine oxidase inhibitors, such as pargyline and clorgyline, nor inhibitors of nitric oxide synthase, such as NG-methyl L-arginine, restored IL-4 production, making the implication of any of these oxidative pathways unlikely.

Thus far, molecules of the cytochrome P450 (CYP450) family remain the most plausible signal carriers, as supported by the following findings. First, 5-HT shares with histamine the capacity to bind to the heme moiety of CYP450.³⁰ Second, a well-known ligand of CYP450 molecules, such as ketoconazole,³¹ exerts a significant inhibition of IL-4 production on its own and competes with the binding of 5-HT because it diminishes the inhibitory

TABLE I. 5-HT-induced inhibition of IL-4 production by basophils is not mediated through SERT

	Stimulation with IL-3	Inhibition (%)
Wild-type mice	+ 5-HT (5×10^{-4} mol/L)	95 $\pm$ 4
	+ 5-HT + FX (10^{-6} mol/L)	95 $\pm$ 3
	+ 5-HT + CTP (10^{-6} mol/L)	94 $\pm$ 6
Sert ^{-/-} mice	+ 5-HT (5×10^{-4} mol/L)	98.7 $\pm$ 1.2

Total BMCs (2.5×10^6 /mL) were incubated for 24 hours in the presence of IL-3 (10 ng/mL) with or without 5-HT. The percentage of inhibition was calculated relative to cultures set up in IL-3 alone. Data are presented as means $\pm$ SEMs from 3 separate experiments.

CTP, Citalopram; FX, fluoxetine.

TABLE II. Biogenic monoamines and OCT3 ligands inhibit basophil activities through 2 distinct mechanisms

Stimulation	Intracellular histamine (ng/10 ⁶ cells)	Inhibition (%)
IL-3	30.0 $\pm$ 8.5	—
+ 5-HT (5×10^{-4} mol/L)	12.5 $\pm$ 3.5	98.3 $\pm$ 1.2
+ CB (10^{-5} mol/L)	55.0 $\pm$ 5.7*	55.3 $\pm$ 0.3
+ CPF (10^{-5} mol/L)	98.5 $\pm$ 4.9†	69.0 $\pm$ 2.0

Basophil-enriched NK1.1⁺CD49b⁺ cells (2.5×10^5 /mL) were incubated for 24 hours in the presence of IL-3 (10 ng/mL) with or without the compounds tested. Histamine levels were measured in cell lysates, and IL-4 levels were measured in supernatants. The percentage of inhibition was calculated relative to cultures in IL-3 alone. Data are presented as means $\pm$ SEMs from 3 separate experiments.

CB, Clobenpropit; CPF, ciproxifan.

**P* = .04.

†*P* = .02.

effect of 5-HT on IL-4 production (see Fig E2 in this article's Online Repository at www.jacionline.org).

Serotonin-induced inhibition of cytokine production by human basophils

Knowing that IL-3 is a poor inducer of T_H2-type cytokine production by human basophils, we performed most experiments with A23187 as a costimulant. Both IL-4 and IL-13 levels were reduced by 5-HT in these conditions (45.3% $\pm$ 6.9% vs 38.3% $\pm$ 6.9% inhibition for IL-13 and IL-4, respectively; means $\pm$ SEMs from 3 donors). Mean IL-13 production in control animals was 910.7 $\pm$ 115.2 versus 483.3 $\pm$ 17.4 pg/10⁶ basophils on exposure to 5-HT (*P* = .003) compared with 4867 $\pm$ 747.4 versus 3103.3 $\pm$ 552 pg of IL-4/10⁶ basophils (*P* = .03). For comparison, purified basophils from one donor were stimulated with IL-3 plus anti-FcεRIα mAb with or without 5-HT. In this case IL-4 and IL-13 were no longer detected on exposure to 5-HT, with control values reaching 276 pg/10⁶ cell for IL-4 and 1037 pg/10⁶ cells for IL-13.

Inhibition of basophil activities by 5-HT and pharmacologic OCT3 inhibitors *in vivo*

The *in vivo* relevance of the functional downregulation of basophils through OCT3 was assessed by using 2 approaches, namely (1) treatment with IL-33, which results in basophil activation,²³ and (2) induction of a basophil-dependent T_H2 response by papain,¹⁸ as evidenced by increased circulating IgE levels. As shown in Fig 4, A, the increased responsiveness of basophils in

terms of IL-4 production conferred by injection of IL-33 was diminished when 5-HT was administered concomitantly. Yet the effect was not as drastic as *in vitro*, probably because the monoamine is rapidly taken up by platelets or metabolized *in vivo*.³² Comparatively, the inhibition by histamine was more consistent yet less pronounced than that obtained with ciproxifan, a synthetic ligand of OCT3 (Fig 4, A). Note that *Oct3*^{-/-} mice produce less IL-4 on stimulation than their wild-type counterparts, indicating that the negative feedback is already operational because of the accumulation of newly synthesized histamine that is no longer secreted through OCT3, as reported before.²² The *in vivo* relevance of OCT3-mediated downregulation of IL-4 production by basophils was further supported by the decrease in circulating IgE levels generated within 14 and 21 days after papain injection when mice had received 5-HT or clobenpropit, another ligand of OCT3, before, during, and shortly after immunization (Fig 4, B). This is the first evidence that the pro-T_H2 functions of basophils and their potential deleterious effect in allergic diseases can be downregulated through OCT3 *in vivo*, either directly or indirectly, by increasing intracellular histamine levels.

DISCUSSION

The adverse effects of basophils in patients with allergic pathologies prompted us to search for negative regulators of their biological activity with potential therapeutic applications. Here we provide evidence that 5-HT and ligands of the transmembrane carrier OCT3³³ downregulate IL-4 production by basophils in response to a variety of stimuli, both *in vitro* and *in vivo*. We demonstrate that the inhibition is mediated through the broad specificity, high-capacity transporter OCT3 rather than specific receptors or the transporter SERT.

It is generally acknowledged that the nervous system modulates the immune response through the release of monoamine neurotransmitters, such as dopamine and serotonin.³⁴ These regulatory functions are believed to involve mainly specific receptors, which comprise several classes and subtypes differentially expressed in hematopoietic populations.^{35,36} The membrane transporters of these monoamines have been regarded essentially as a means of rapid reuptake to prevent excessive accumulation in the vicinity of the neurons from which they are released.³⁷ However, monoamine reuptake systems, such as SERT, are also expressed outside the central nervous system in various hematopoietic cells, such as platelets, lymphocytes, and macrophages.^{38,39} In such cells transmembrane carriers can take part in more complex regulatory activities, the effect of which on the immune response deserves further investigation.^{40,41} For example, it has been reported that dendritic cells take up 5-HT through the monoamine transporter SERT and store it in lysosomal-associated membrane protein 1 vesicles, from which it can eventually be released through Ca²⁺-dependent exocytosis.⁴²

We have previously reported that newly synthesized histamine accumulating inside basophils can downregulate their typical functions.²² Given the bidirectional activity of OCT3, we assumed that 5-HT inhibited basophil functions by blocking the transporter, thus hampering the release of newly generated histamine, such as other substrates or inhibitors of OCT3 (eg, clobenpropit, corticosterone, or β-estradiol).⁴³ This was not the case because 5-HT neither increased the histamine content of basophils stimulated with IL-3 nor was its effect abolished in histamine-free *Hdc*^{-/-} mice.

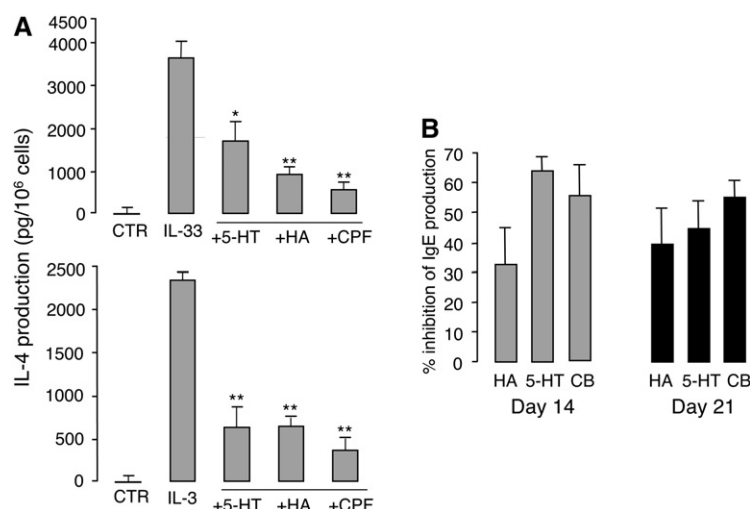


FIG 4. OCT3 ligands decrease basophil functions *in vivo*. **A**, BMCs from mice receiving daily injections of IL-33 for 4 days with or without coinjected histamine (HA), serotonin (5-HT), or ciproxifan (CPF) were recovered on day 4 and restimulated with IL-33 (10 ng/mL) for 24 hours compared with saline controls (n = 3). **B**, IgE levels were measured after immunization with papain in sera of mice treated or not with OCT3 ligands (n = 3). CB, Clobenpropit. **P* ≤ .01; ***P* ≤ .002.

Like histamine, 5-HT inhibits IL-4 production by basophils on transcriptional and protein levels, most likely by signaling through the CYP450 pathway. Molecules of this family are expressed in bone marrow, where they were supposed to be mainly involved in detoxification and drug-metabolizing processes. However, it has also been established that they constitute a branch of the arachidonic acid cascade or third pathway, and some of their metabolites can contribute to the regulation of hematopoiesis and cytokine expression.^{44,45} This is particularly true for prostanoids, which affect the T_H1/T_H2 balance by inhibiting IL-4 production.⁴⁶ Other mechanisms of functional modulation, such as serotonylation of proteins involved in signal transduction, might also come into play.⁴⁷

Note that the inhibitory effect of histamine or 5-HT mediated through OCT3 is not observed in mast cells, the other major histamine-producing population. We ignore whether the negative regulation of basophil functions by 5-HT might be of physiological relevance during inflammatory responses when mast cell degranulation and platelet aggregation provide locally high levels of this biogenic amine. However, we postulate that pharmacologic targeting of OCT3 could become instrumental in tuning the regulatory functions of this population to alleviate its detrimental effects or to modulate its effect on the orientation of the immune response. Our *in vivo* data support a therapeutic potential for this approach because the administration of monoamines or ciproxifan downregulated (1) the enhanced IL-4-producing capacity of basophils in mice having received the proinflammatory cytokine IL-33 and (2) the IgE production induced by papain in a model of basophil-dependent T_H2 differentiation,¹⁸ and (3) a similar inhibition of IL-4 and IL-13 production occurred in stimulated human basophils. The increased *in vivo* efficiency of this synthetic inhibitor relative to biogenic monoamines that are easily metabolized or taken up by other cells is promising from a therapeutic point of view, supporting the conclusion that OCT3 might become a pharmacologic target in some allergic disorders.

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Clinical implications: We postulate that the membrane transporter OCT3 might become a therapeutic target for tuning the activity of basophils, either to attenuate basophil-mediated pathologies or to control the T_H1/T_H2 balance.

REFERENCES

- Schneider E, Thieblemont N, Leite De Moraes M, Dy M. Basophils: new players in the cytokine network. *Eur Cytokine Netw* 2010;21:142-53.
- Min B, Paul WE. Basophils and type 2 immunity. *Curr Opin Hematol* 2008;15: 59-63.
- Karasuyama H, Mukai K, Tsujimura Y, Obata K. Newly discovered roles for basophils: a neglected minority gains new respect. *Nat Rev Immunol* 2009;9:9-13.
- Mukai K, Matsuoka K, Taya C, Suzuki H, Yokozeki H, Nishioka K, et al. Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity* 2005;23:191-202.
- Schneider E, Lemoine FM, Breton-Gorius J, Machavoine F, Arnould A, Cramer EM, et al. IL-3-induced coexpression of histidine decarboxylase, IL-4 and IL-6 mRNA by murine basophil precursors. *Exp Hematol* 1999;27:1010-8.
- Dy M, Schneider E, Debili N, Dumenil D, Pacilio M, Rolli-Derkinderen M, et al. Histidine decarboxylase activity in hematopoietic progenitors as a marker for basophilic potentiality. In: Watanabe T, Timmerman H, Yanai K, editors. *Histamine research in the new millennium*. Amsterdam: Elsevier; 2001. p. 319-24.
- Dy M, Jankovic D, Ploemacher RE, Theze J, Schneider E. Concomitant histamine, interleukin 4 and interleukin 6 production by hematopoietic progenitor subsets in response to interleukin 3. *Exp Hematol* 1991;19:934-8.
- Salachas F, Schneider E, Lemoine F, Navarro S, Lebel B, Ziltener H, et al. Aggregated IgE-induced histamine synthesis by murine hematopoietic progenitors. *Blood* 1994;84:1098-107.
- Mitre E, Taylor RT, Kubofcik J, Nutman TB. Parasite antigen-driven basophils are a major source of IL-4 in human filarial infections. *J Immunol* 2004;172:2439-45.
- Min B, Prout M, Hu-Li J, Zhu J, Jankovic D, Morgan ES, et al. Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J Exp Med* 2004;200:507-17.
- Mukai K, Obata K, Tsujimura, Karasuyama H. New insights into the roles for basophils in acute and chronic allergy. *Allergol Int* 2009;58:11-9.
- Devouassoux G, Foster B, Scott LM, Metcalfe DD, Prussac C. Frequency and characterization of antigen-specific IL-4- and IL-13-producing basophils and T cells in peripheral blood of healthy and asthmatic subjects. *J Allergy Clin Immunol* 1999; 104:811-9.
- Kepley C, McFeeley PJ, Oliver JM, Lipscomb MF. Immunohistochemical detection of human basophils in postmortem cases of fatal asthma. *Am J Respir Crit Care Med* 2001;164:1053-8.

14. Khodoun MV, Orekhova T, Potter C, Morris S, Finkelman FD. Basophils initiate IL-4 production during a memory T-dependent response. *J Exp Med* 2004;200:857-70.
15. Moore ML, Newcomb DC, Parekh VV, Van Kaer L, Collins RD, Zhou W, et al. STAT1 negatively regulates lung basophil IL-4 expression induced by respiratory syncytial virus infection. *J Immunol* 2009;183:2016-26.
16. Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat Immunol* 2008;9:310-8.
17. Wada T, Ishiwata K, Koseki H, Ishikura T, Ugajin T, Ohnuma N, et al. Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. *J Clin Invest* 2010;120:2867-75.
18. Sokol CL, Chu NQ, Yu S, Nish SA, Laufer TM, Medzhitov R. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat Immunol* 2009;10:713-20.
19. Perrigoue JG, Saenz SA, Siracusa MC, Allenspach EJ, Taylor BC, Giacomini PR, et al. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat Immunol* 2009;10:697-705.
20. Yoshimoto T, Yasuda K, Tanaka H, Nakahira M, Imai Y, Fujimori Y, et al. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat Immunol* 2009;10:706-12.
21. Sohee K, Shen T, Min B. Basophils can directly present or cross-present antigen to CD8 lymphocytes and alter CD8 T cell differentiation into IL-10-producing phenotypes. *J Immunol* 2009;183:3033-9.
22. Schneider E, Machavoine F, Pléau JM, Bertron AF, Thurmond RL, Ohtsu H, et al. Organic cation transporter 3 modulates murine basophil functions by controlling intracellular histamine levels. *J Exp Med* 2005;202:387-93.
23. Schneider E, Petit-Bertron AF, Bricard R, Levasseur M, Ramadan A, Girard JP, et al. IL-33 activates unprimed murine basophils directly in vitro and induces their in vivo expansion indirectly by promoting hematopoietic growth factor production. *J Immunol* 2008;183:3591-7.
24. Ohtsu H, Tanaka S, Terui T, Hori Y, Makabe-Kobayashi Y, Pejler G, et al. Mice lacking histidine decarboxylase exhibit abnormal mast cells. *FEBS Lett* 2001;502:53-6.
25. Bengel D, Murphy DL, Andrews AM, Wichems CH, Feltner D, Heils A, et al. Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter-deficient mice. *Mol Pharmacol* 1998;53:649-55.
26. Zwart R, Verhaagh S, Buitelaar M, Popp-Snijders C, Barlow DP. Impaired activity of the extraneuronal monoamine transporter system known as uptake-2 in Oct3/Slc22a3-deficient mice. *Mol Cell Biol* 2001;21:4188-96.
27. Croker BA, Kiu H, Nicholson SE. SOCS regulation of the JAK/STAT signaling pathway. *Semin Cell Dev Biol* 2008;19:414-22.
28. Chen YH, Bieneman AP, Creticos PS, Chichester KL, Schroeder JT. IFN- α inhibits IL-3 priming of human basophil cytokine secretion but not leukotriene C4 and histamine release. *J Allergy Clin Immunol* 2003;112:944-50.
29. Liu H, Colavitti R, Rovira II, Finkel T. Redox-dependent transcriptional regulation. *Circ Res* 2005;97:967-74.
30. LaBella FS, Brandes LG. Interaction of histamine and other bioamines with cytochromes P450: implications for cell growth modulation and chemopotentiality by drugs. *Semin Cancer Biol* 2000;10:47-53.
31. Pal D, Mitra AK. MDR- and CYP3A4-mediated drug-drug interactions. *J Neuroimmune Pharmacol* 2006;1:323-39.
32. Lesch KP, Wolozin BL, Murphy DL, Reiderer P. Primary structure of the human platelet serotonin uptake site: identity with the brain serotonin transporter. *J Neurochem* 1993;60:2319-22.
33. Koepsell H, Lips K, Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharm Res* 2007;24:1227-51.
34. Meredith EJ, Chamba A, Holder MJ, Barnes NM, Gordon J. Close encounters of the monoamine kind: immune cells betray their nervous disposition. *Immunology* 2005;115:289-95.
35. Idzko M, Panther E, Stratz C, Müller T, Bayer H, Zissel G, et al. The serotonergic receptors of human dendritic cells: identification and coupling to cytokine release. *J Immunol* 2004;172:6011-9.
36. Léon-Ponte M, Ahern GP, O'Connell PJ. Serotonin provides an accessory signal to enhance T-cell activation by signaling through the 5-HT7 receptor. *Blood* 2007;109:3139-46.
37. Fox MA, Andrews AM, Wendland JR, Lesch KP, Holmes A, Murphy DL. A pharmacological analysis of mice with a targeted disruption of the serotonin transporter. *Psychopharmacology* 2007;2:147-66.
38. Gordon J, Barnes NM. Lymphocytes transport serotonin and dopamine: agony or ecstasy? *Trends Immunol* 2003;24:438-43.
39. Rudd ML, Nicolas AN, Brown BL, Fischer-Stenger K, Stewart JK. Peritoneal macrophages express the serotonin transporter. *J Neuroimmunol* 2005;159:113-8.
40. Boehme SA, Lio FM, Sikora L, Pandit TS, Lavrador K, Rao SP, et al. Cutting edge: serotonin is a chemotactic factor for eosinophils and functions additively with eotaxin. *J Immunol* 2004;173:3599-603.
41. Van Loveren H, Den Otter W, Meade R, Terheggen PM, Askenase PW. A role for mast cells and the vasoactive amine serotonin in T cell-dependent immunity to tumors. *J Immunol* 198;134:1292-9.
42. O'Connell PJ, Wang X, Leon-Ponte M, Griffiths C, Pingle SC, Ahern GP. A novel form of immune signaling revealed by transmission of the inflammatory mediator serotonin between dendritic cells and T cells. *Blood* 2006;107:1010-7.
43. Gründemann D, Liebich G, Kiefer N, Köster S, Schömig E. Selective substrates for non-neuronal monoamine transporters. *Mol Pharmacol* 1999;56:1-10.
44. Lutton JD, Levere RD, Abraham NG. Physiologic role of heme and cytochrome P-450 in hematopoietic cells. *Proc Soc Exp Biol Med* 1991;196:260-9.
45. Sasaki M, Elrod JW, Jordan P, Itoh M, Joh T, Minagar A, et al. CYP450 dietary inhibitors attenuate TNF- α -stimulated endothelial molecule expression and leukocyte adhesion. *Am J Physiol Cell Physiol* 2004;286:C931-9.
46. Parker CW, Huber MG, Godt SM. Modulation of IL-4 production in murine spleen cells by prostaglandins. *Cell Immunol* 1995;160:278-85.
47. Walther DJ, Peter JU, Winter S, Hölte M, Paulmann N, Grohmann M, et al. Serotonylation of small GTPases is a signal transduction pathway that triggers platelet α -granule release. *Cell* 2003;115:851-62.

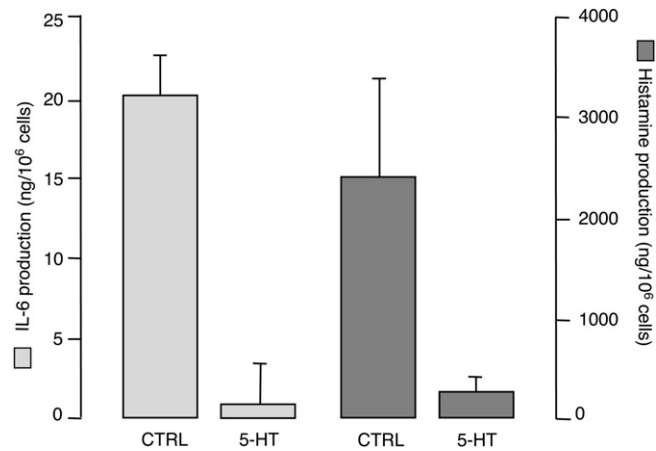


FIG E1. 5-HT inhibits IL-3–induced IL-6 and histamine production. Basophil-enriched NK1.1⁺ CD49b⁺ cells (2.5×10^5 /mL) were incubated for 24 hours in the presence of IL-3 (10 ng/mL) with or without 5-HT (5×10^{-4} mol/L). Data are presented as means $\pm$ SEMs from 4 separate experiments. *CTRL*, Control.

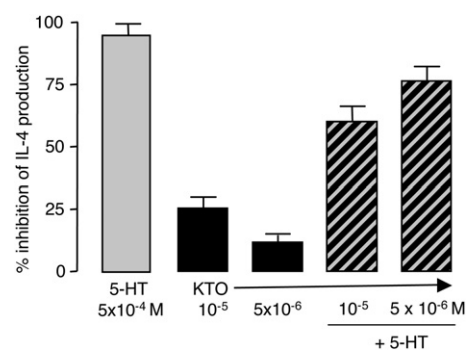


FIG E2. The CYP450 ligand ketoconazole (*KTO*) inhibits IL-3-induced IL-4 production by basophils and competes with 5-HT ($n = 3$).