Production of Serotonin by Tryptophan Hydroxylase 1 and Release via Platelets Contribute to Allergic Airway Inflammation

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***Rationale*: 5-Hydroxytryptamine (5-HT) is involved in the pathogenesis of allergic airway inflammation (AAI). It is unclear, however, how 5-HT contributes to AAI and whether this depends on tryptophan hydroxylase (TPH) 1, the critical enzyme for peripheral 5-HT synthesis.**
***Objectives*: To elucidate the role of TPH1 and the peripheral source of 5-HT in asthma pathogenesis.**
***Methods*: TPH1-deficient and TPH1-inhibitor–treated animals were challenged in ovalbumin and house dust mite models of AAI. Experiments with bone marrow chimera, mast cell–deficient animals, platelets transfusion, and bone marrow dendritic cells (BMDC) driven model of AAI were performed. 5-HT levels were measured in bronchoalveolar lavage fluid or serum of animals with AAI and in human asthma.**
***Measurements and Main Results*: 5-HT levels are increased in bronchoalveolar lavage fluid of mice and people with asthma after allergen provocation. TPH1 deficiency and TPH1 inhibition reduced all cardinal features of AAI. Administration of exogenous 5-HT restored AAI in TPH1-deficient mice. The pivotal role of 5-HT production by structural cells was corroborated by bone marrow chimera experiments. Experiments in mast cell–deficient mice revealed that mast cells are not a source of 5-HT, whereas transfusion of platelets from wild-type and TPH1-deficient mice revealed that only platelets containing 5-HT enhanced AAI. Lack of endogenous 5-HT *in vitro* and *in vivo* was associated with an impaired Th2-priming capacity of BMDC.**
***Conclusions*: In summary, TPH1 deficiency or inhibition reduces AAI. Platelet- and not mast cell–derived 5-HT is pivotal in AAI, and lack of 5-HT leads to an impaired Th2-priming capacity of BMDC. Thus, targeting TPH1 could offer novel therapeutic options for asthma.**

Keywords: serotonin; tryptophan hydroxylase 1; asthma; platelets; dendritic cells

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Recent evidence suggests a role of serotonin (5-HT) in the pathogenesis of asthma. However, the precise source of endogenous 5-HT release in the lungs during allergic airway inflammation (AAI) and its potential role in dendritic cell biology in AAI are unknown.

What This Study Adds to the Field

5-HT levels are elevated in the airways of humans and mice with AAI. Lack of 5-HT by genetic and pharmacologic inhibition of TPH1 attenuates AAI. Mechanistically, 5-HT plays a role in dendritic cell activation.

Allergic asthma is one of the most common chronic diseases worldwide. Clinically, it is characterized by variable airway obstruction, excessive mucus production, and airway hyperresponsiveness (www.ginasthma.com). Chronic airway inflammation is orchestrated by inflammatory cells, such as eosinophils, mast cells, Th2 lymphocytes, and dendritic cells (DCs), resulting in airway remodeling, which is closely associated with the degree of airway obstruction and hyperresponsiveness (1). Despite increased knowledge of the underlying mechanism of allergic airway disease, novel treatment strategies are scarce and so far a cure is not possible.

Serotonin (5-HT) is one of the best characterized neurotransmitters and vasoactive amines involved in the regulation of various physiologic functions including appetite, sleep, mood, and pain (2). Thus far, 15 different 5-HT receptor subtypes have been identified. Besides its major role in central nervous homeostasis, 5-HT has also emerged as an important inflammatory mediator in the peripheral immune system (3–5). Of note, serotonin has been shown to modulate adhesion, migration, and cytokine-chemokine production in cells that are classically also involved in asthma, such as mast cells, eosinophils, monocytes and macrophages, DCs, lung epithelial, and lung fibroblasts (6–14).

Most 5-HT in the body is produced in the periphery (>95%) by intestinal enterochromaffine (EC) cells (15). It has long been known that tryptophan hydroxylase (TPH) catalyzes 5-HT synthesis from its precursor L-tryptophan, but recently two TPH isoforms have been identified: TPH1, which is expressed in nonneuronal cells,

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such as EC cells, spleen, mast cells, and T lymphocytes; and the TPH2 isoform, which is expressed in the raphe nuclei of the brainstem and myenteric plexus neurons (13, 16, 17). After release from EC cells into blood plasma, 5-HT is rapidly taken up by resting platelets via the 5-HT–specific transporter and stored in their dense granules at millimolar concentrations (18). In turn, 5-HT can be released from circulating platelets and resident mast cells after stimulation by inflammatory signals, such as platelet-activating factor, complement components, and IgE complexes or general platelet activation (17, 19, 20).

Several observations suggest that 5-HT may play an important role in the pathogenesis of allergic asthma. Elevated plasma 5-HT levels were found in symptomatic patients with asthma, correlating with clinical severity and pulmonary function (21). Accordingly, in a pilot study treatment of patients with asthma with the platelet 5-HT reuptake accelerator tianeptine resulted in clinical improvement (22–24). Furthermore pharmacologic blockade of 5-HT2 receptor subtypes attenuated the development of allergic airway inflammation (AAI) and remodeling in mice (25, 26).

However, the role of TPH1 in the context of AAI is unknown. Furthermore, the precise source of endogenous 5-HT in AAI and whether this mediator accumulates in the lung after allergen challenge in mice and humans has not been elucidated. Here we provide evidence that 5-HT accumulates in the airways of subjects with asthma and in mice with experimental asthma after allergen challenge. Furthermore, we show that genetic and pharmacologic inhibition of TPH1 attenuates AAI. Interestingly, platelet-derived and not mast cell–derived serotonin is pivotal in AAI. Finally, TPH1-derived serotonin contributes to the allergen-specific activation and function of bone marrow dendritic cells (BMDCs). Together, these data show that platelet serotonin contributes to AAI and that endogenous serotonin is involved in regulating BMDC-induced Th2 immunity.

METHODS

Mice

C57/Bl6 wild-type (WT) animals, TPH1-deficient mice ($TPH1^{-/-}$), and ovalbumin (OVA)–T cell receptor transgenic OT-II mice on a C57/Bl6 background, which were generated as previously described (16), were bred at the University of Freiburg. Genetically mast cell–deficient KitW-sh/KitW-sh mice and the congenic Kit^+/Kit^+ WT littermates were obtained by intercrossing heterogeneous KitW-sh/Kit^+ mice at the University of Mainz (27). All experiments were performed according to institutional guidelines of the animal ethics committee from the German government.

OVA-Alum Model of AAI

Mice were sensitized to and challenged with OVA as previously described (28). One day after the last OVA challenge airway hyperresponsiveness, bronchoalveolar lavage (BAL) cell differentiation, lung resection for histology, and Th2 cytokines levels in restimulated mediastinal lymph nodes (MLNs) were determined as previously described (29). For details *see* the online supplement.

Generation of BM Chimera

WT or $TPH1^{-/-}$ recipients (both C57Bl/6) were given 5×10^6 WT or $TPH1^{-/-}$ BM cells (C57Bl/6) intravenously after lethal irradiation with 900 cGy (2×450 cGy). The following donor–recipient pairs were combined: WT→WT; $TPH1^{-/-}$→WT (hematopoietic system: $TPH1^{-/-}$); WT→$TPH1^{-/-}$ (nonhematopoietic system: $TPH1^{-/-}$); and $TPH1^{-/-}$→$TPH1^{-/-}$.

Experiments with Mast Cell–Deficient Mice

OVA or sham sensitized and OVA-challenged KitW-sh/KitW-sh and congenic Kit^+/Kit^+ animals were analyzed 1 day after the last OVA exposure and the classical features of AAI were determined as described in the online supplement.

Platelet Transfusion

Purified platelets (total of 2×10^7 platelets per mouse) from WT or $TPH1^{-/-}$ donor mice were transfused into WT or $TPH1^{-/-}$ recipient mice on Days 0, 5, 10, 15, and 20 after sensitization with OVA. For details, *see* the online supplement.

Measurement of 5-HT Levels

5-HT levels in the BAL fluid (BALF) and cell supernantant, platelet-free plasma, and serum were quantified by enzyme immunoassay according to the manufacturer's instructions (Labor Diagnostika Nord, Nordhorn, Germany) as described in the online supplement.

Th2 Sensitization Induced by Intratracheal Injection of OVA-pulsed DCs

DCs were prepared as previously described (28). For details, *see* the online supplement. C57Bl/6 mice were anesthetized on Day 0 with ketamine-xylazine, and 1×10^6 vehicle-WT-DCs, OVA-WT-DCs, vehicle-$TPH1^{-/-}$-DCs, or OVA-$TPH1^{-/-}$-DCs were instilled intratracheally through the vocal cords as described (28). On Days 10 through 12, mice were exposed to OVA aerosols (1% for 30 min). Mice were killed 24 hours after the last aerosol administration.

Segmental Allergen Provocation

Segmental allergen provocation in patients with mild allergic asthma was performed as previously described (30–32). The study was approved by the local ethics committee. For details and patient characteristic, *see* Tables E1 and E2 in the online supplement.

Flow Cytometry

BAL cell differentiation and the DC maturation were determined by flow cytometry as described previously (28, 33). For details, *see* the online supplement.

Statistical Analysis

Values for all measurements are expressed as the mean ± SEM. Unless stated otherwise, the statistical significance of differences between samples was calculated using analysis of variance, followed by Bonferroni comparison test. Differences were considered significant if P was less than 0.05.

RESULTS

Increased 5-HT Levels in BALF of Animals and Patients with Acute Asthmatic Airway Inflammation

To test whether 5-HT accumulates during AAI in mice and humans, BALF from OVA-sensitized and -challenged mice or patients with allergic asthma 24 hours after segmental challenge with saline or allergen (*see* Table E1 for patient characteristics and Table E2 for cell counts in BAL) was collected. As shown in Figure 1 allergen challenge in sensitized mice and humans with asthma led to a significant increase in BALF 5-HT levels (Figures 1A and 1B).

Of note, in patients with asthma, allergen provocation was associated with a significant decrease in serum 5-HT levels (Figure 1C), suggesting that 5-HT translocates from platelet stores into the lung after allergen provocation. Interestingly, a significant increase in BALF 5-HT levels could also be observed in stable subjects with asthma compared with healthy control subjects (Figure 1D).

Reduced AAI in $TPH1^{-/-}$ Mice

To better define the role of 5-HT in the pathogenesis of AAI, $TPH1^{-/-}$ mice and WT control animals were sham- (phosphate-buffered

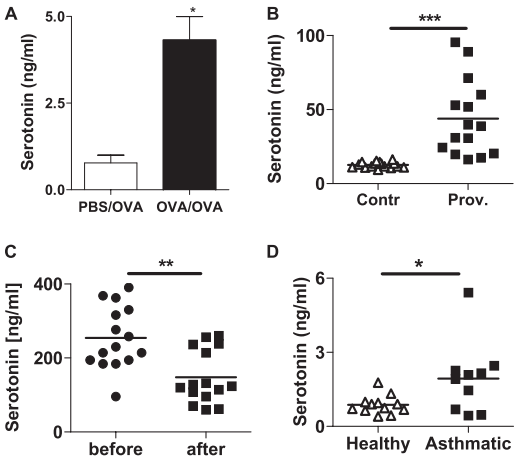


Figure 1. Accumulation of serotonin (5-HT) level in the bronchoalveolar lavage fluid (BALF) during allergic airway inflammation in mice and humans. (*A*) Mice were sensitized by intraperitoneal injection of ovalbumin (OVA)/alum on Days 0 and 7 and were exposed to OVA aerosols on Days 19–21. BALF was collected and free 5-HT levels were measured by ELISA. Data are mean ± SEM, n = 8 mice. *$P < 0.001$. One experiment out of three independent is shown. (*B*) Patients with asthma underwent segmental allergen provocation and levels of 5-HT in BALF were detected 24 hours after saline (Contr.) or allergen (Prov.) challenge. (*C*) Serum 5-HT levels were measured before and 24 hours after segmental allergen provocation. Data are mean ± SEM; n = 15 patients. ***$P < 0.0006$, **$P < 0.001$. (*D*) BALF was collected from healthy control subjects (n = 12) and stable patients with asthma (n = 10) and free 5-HT levels were measured by ELISA. *$P < 0.05$. PBS = phosphate-buffered saline.

saline/alum) or OVA-sensitized (OVA/alum) and challenged with OVA aerosols. OVA-sensitized and -challenged WT animals showed a marked eosinophilia and increased 5-HT levels in the BALF (Figures 2A and 2C), enhanced Th2 cytokine production by MLN cells (Figure 2D), and peribronchial and perivascular inflammation (Figure 2E), which was not detectable in sham-sensitized mice. The lack of endogenous 5-HT in blood and BALF of OVA-sensitized and -challenged $TPH1^{-/-}$ mice (Figures 2A and 2B) was associated with a significant reduction in BALF eosinophila (Figure 2C), peribronchial and perivascular tissue infiltration (Figure 2E), and Th2 cytokine levels (IL-4, IL-5, and IL-13) in restimulated MLN cells (Figure 2D). Of note, in $TPH1^{-/-}$ mice also the bronchial hyperresponsiveness to methacholine, determined by invasive measurement of dynamic resistance and compliance, was significantly reduced (Figure 2F).

To further validate the role of $TPH1^{-/-}$ in AAI, OVA-sensitized WT animals were treated with the TPH1-inhibitor *p*-chlorophenylalanine (PCPA). As demonstrated in Figure 2G, PCPA significantly decreased BALF 5-HT levels (Figure 2G), which was accompanied by a reduction in BALF eosinophils, peribronchial and perivascular tissue infiltration, and Th2 cytokines (Figures 2H–2J). Together, these findings show that genetic and pharmacologic inhibition of TPH1 significantly reduces AAI.

To confirm that the reduced asthmatic phenotype in $TPH1^{-/-}$ animals was caused by a lack of nonneuronal 5-HT, we restored 5-HT levels in OVA-sensitized $TPH1^{-/-}$ animals by intraperitoneal administration of exogenous 5-HT. As shown in Figures 2K and 2L administration of 5-HT restored AAI in $TPH1^{-/-}$ animals close to WT levels.

TPH1 Expression in Structural Cells Is Required for AAI

Because not only intestinal EC cells (structural cells) but also immune cells, such as mast cells, and T lymphocytes (hematopoietic cells) express TPH1 (16, 17, 34, 35), $TPH1^{-/-}$ and WT BM chimeras were sensitized and challenged with OVA. As shown in Figures 3A–3D, the lack of TPH1–5-HT in the recipient (WT→$TPH1^{-/-}$) but not in the donor ($TPH1^{-/-}$→WT) led to decreased BAL 5-HT levels, which was accompanied with attenuated BAL eosinophilia and Th2 cytokine levels in MLNs. This indicates that nonhematopoietic (i.e., most likely EC cell–derived TPH1–5-HT) is required for the full AAI response. In keeping with this finding high serum 5-HT levels were only observed in WT but not in the $TPH1^{-/-}$ recipients (Figure 3E).

Mast Cells Are Not the Primary Source of 5-HT in AAI

Resident mast cells in the lung may not have been entirely eliminated by the irradiation in the aforementioned BM transplantation experiments. Thus, to exclude the involvement of mast cell–derived 5-HT in our model, mast cell–deficient ($Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$) and WT ($Kit^{+}/Kit^{+}$) animals were OVA sensitized and challenged. As shown in Figure 3, BALF 5-HT levels in sensitized and challenged $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ were comparable with those of Kit^{+}/Kit^{+} animals (Figures 3F and 3G), suggesting that mast cells are not the primary source of 5-HT in AAI.

Platelets Are the Main Source of 5-HT in AAI

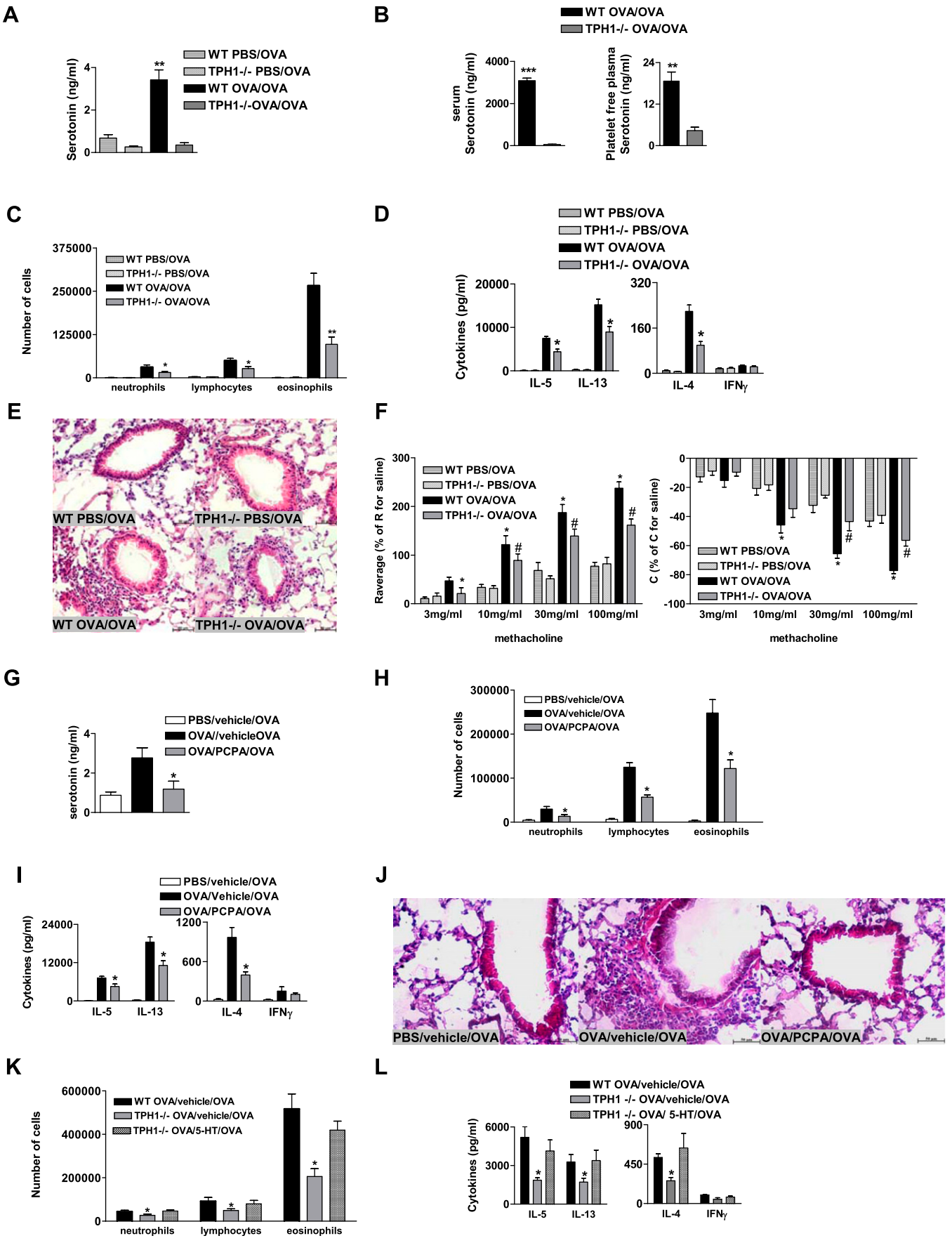
Platelets are the major storage vehicles for EC cell–derived 5-HT. To assess if platelet-released 5-HT contributes to AAI, experiments with on-top transfusion of platelets from WT (i.e., 5-HT–containing platelets) (18) or $TPH1^{-/-}$ mice (i.e., 5-HT–free platelets) (16, 36) was performed. Repeated transfusion of WT platelets into sensitized and OVA-challenged WT recipient resulted in a slight but not significant increase in BAL eosinophilia compared with untransfused WT mice (Figure 4A). Transfusion of $TPH1^{-/-}$ platelets into WT recipients did not alter BALF eosinophila, Th2 cytokine production, or tissue infiltration compared with untransfused WT mice (Figures 4A–4C). Transfusion of WT platelets into $TPH1^{-/-}$ recipients almost completely restored all features of AAI (Figures 4A–4C). In contrast, $TPH1^{-/-}$ mice receiving $TPH1^{-/-}$ platelets had a significantly reduced AAI compared with WT animals. Of note, BALF 5-HT levels increased after transfusion of WT platelets but not after TPH1 platelets (Figure 4D). These results illustrate the importance of platelet-derived 5-HT for the development of AAI.

DC from $TPH1^{-/-}$ Mice Display a Distorted Maturation, Cytokine Production, and Reduced Th2 Priming Capacity

Because activation of DCs is an essential step in AAI and 5-HT has been shown to modulate migration, cytokine release, and the Th2 priming capacity of DCs *in vitro* (12), the importance of TPH1 in BMDCs was investigated. Analysis of the maturation and costimulatory molecules revealed that $TPH1^{-/-}$ BMDCs showed a slight reduction in CD40, CD80, and CD86 expression compared with $TPH1^{+/+}$ DCs (*see* Table E3). In addition, OVA-pulsed $TPH1^{-/-}$ DCs produced lower levels of IL-1β and IL-12 (Figure 5A).

Next, the ability of OVA DCs to prime OVA–T cell receptor–OT II CD4-T cells *in vivo* was tested. As shown in Figure 5B, OT II cells of mice immunized with $TPH1^{-/-}$ OVA DC produced lower levels of IL-4, IL-5, and IL-13 compared with cells stimulated with $TPH1^{+/+}$ OVA DCs (Figure 5B).

To further define the relevance of TPH1 for the Th2 priming capacity of BMDC *in vivo*, OVA-pulsed $TPH1^{-/-}$ DCs and WT DCs were given intratracheally to $TPH1^{-/-}$ or WT mice. As demonstrated in Figures 5C–5E, the application OVA-pulsed WT DCs into WT animals induces BAL eosinophila, peribronchial and perivascular


A
Serotonin (ng/ml)
WT PBS/OVA
TPH1-/- PBS/OVA
WT OVA/OVA
TPH1-/-OVA/OVA
B
WT OVA/OVA
TPH1-/- OVA/OVA
serum Serotonin (ng/ml)
Platelet free plasma Serotonin (ng/ml)
C
Number of cells
neutrophils
lymphocytes
eosinophils
D
Cytokines (pg/ml)
IL-5
IL-13
IL-4
IFNγ
E
WT PBS/OVA
TPH1-/- PBS/OVA
WT OVA/OVA
TPH1-/- OVA/OVA
F
Raverage (% of R for saline)
C (% of C for saline)
3mg/ml
10mg/ml
30mg/ml
100mg/ml
methacholine
G
serotonin (ng/ml)
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TPH1-/- OVA/5-HT/OVA
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TPH1 -/- OVA/ 5-HT/OVA
Cytokines (pg/ml)

Figure 2. Genetic and pharmacologic interference with tryptophan hydroxylase (TPH)-1$^{-/-}$ is associated with reduced allergic airway inflammation. (*A–F*) Wild-type (WT) and TPH1$^{-/-}$ mice were sensitized by intraperitoneal injection of ovalbumin (OVA) at Days 0 and 7 and exposed to OVA aerosols on Days 19–21. Serotonin (5-HT) levels in bronchoalveolar lavage fluid (BALF) (*A*) and serum and plasma (*B*) were detected by ELISA. (*C*) BALF cell differential counts were measured by flow cytometry. (*D*) Cytokine production in mediastinal lymph node (MLN) cells restimulated *in vitro* for 4 days with OVA was assessed by ELISA. (*E*) Hematoxylin and eosin (H&E) staining of lung sections. Data are mean ± SEM; n = 6–8. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (*F*) Bronchial hyperresponsiveness to various doses of aerosolized methacholine 24 hours after the last antigen exposure were measured by changes in average resistance ("R") and lung compliance ("C") in mechanically ventilated mice. Data are mean ± SEM; n = 8. *$P < 0.01$, phosphate-buffered saline (PBS)/OVA versus OVA/OVA; $^{\#}P < 0.05$, TPH1$^{+/+}$ OVA/OVA versus TPH1$^{-/-}$ OVA/OVA. (*G–J*) WT mice were challenged as described previously and treated with the TPH inhibitor PCPA or vehicle as indicated. Groups are coded as sensitization/treatment/challenge. BALF 5-HT levels (*G*), cell populations (*H*), and cytokine production in MLN cells restimulated *in vitro* for 4 days with OVA (*I*) were measured as described previously. (*J*) H&E staining of lung sections. Data are mean ± SEM; n = 6–8 mice in each group. *$P < 0.01$. One experiment out of three independent is shown. (*K* and *L*) TPH1$^{-/-}$ mice received an intraperitoneal injection of 5-HT at Days 18–21 and were challenged as described previously. BALF cell differential counts (*K*) and cytokine production in MLN cells restimulated *in vitro* for 4 days with OVA (*L*) were measured as described previously. Data are mean ± SEM; n = 6–8 mice in each group. *$P < 0.01$. One experiment out of three independent is shown.

inflammation, and the production of the Th2 cytokines by MLNs. In WT or TPH1$^{-/-}$ animals receiving OVA-TPH1$^{-/-}$ DCs these features of AAI were significantly lower (Figures 5C–5E). However, when WT-OVA DCs were injected into TPH1$^{-/-}$ mice no significant differences, apart from a trend toward a reduction in the number of BAL eosinophils, was detected.

To elucidate whether the distorted maturation and T-cell priming capacity of TPH1$^{-/-}$ BMDCs could be explained by a lack of 5-HT, we supplemented the cell culture medium with 5-HT in concentrations comparable with those achieved by local release after platelet activation (37). TPH1$^{-/-}$ DC cultured in the 5-HT–supplemented medium showed a very similar expression pattern of CD40, CD80, CD83, and CD86 compared WT DC after OVA-pulsing (*see* Table E4). Moreover, the Th2 priming capacity of 5-HT–treated OVA-TPH1$^{-/-}$ DCs was comparable with those of OVA-WT DCs (Figure 5F).

To determine whether significant levels of 5-HT accumulate in BM, the amount of 5-HT in freshly isolated BM cells from WT and TPH1$^{-/-}$ animals was measured. As shown in Figure 5G, BM cells from WT but not from TPH1$^{-/-}$ mice contain high levels of 5-HT. Finally, we examined whether endogenous 5-HT delivered to BM cells is pivotal for the capacity of BMDC to prime for Th2 immunity *in vivo*. To do so, BM from TPH1$^{-/-}$ mice was transferred into irradiated WT recipients (TPH1$^{-/-}$ BM/WT) or TPH1$^{-/-}$ recipients (TPH1$^{-/-}$ BM/TPH1$^{-/-}$), and BM from WT mice was transferred to irradiated TPH1$^{-/-}$ recipients (WT BM/ TPH1$^{-/-}$) or WT mice (WT BM/WT). OVA-pulsed BMDC generated from TPH1$^{-/-}$ BM/WT animals showed similar expression of the costimulatory molecules CD40, CD80, CD83, and CD86 compared with OVA-pulsed WT BM/WT mice (*see* Table E5). Accordingly, when WT BM/ TPH1$^{-/-}$ OVA-pulsed DCs were given intratracheally to WT mice, no significant differences in the number of BAL eosinophila could be observed compared with WT mice, which received OVA-pulsed WT BM/WT BMDCs (Figure 5H). In contrast, OVA-pulsed DCs from TPH1$^{-/-}$ animals that were reconstituted with WT-BM (WT BM/TPH1$^{-/-}$ OVA DCs) still showed an impaired maturation and Th2 priming capacity (Figure 5H; *see* Table E5). Taken together these findings suggest that during their generation, BMDCs require activation by 5-HT for full maturation and Th2 priming response.

DISCUSSION

Although previous studies suggest a role of 5-HT in the pathogenesis of AAI, it has remained unclear which cells mediate 5-HT–enhanced hyperresponsiveness (6, 21, 25, 26). Knowing the exact mechanism by which 5-HT is released locally and characterization of its target cells might allow the development of tailored therapies, which would not interfere with other important functions of 5-HT, thus limiting possible side effects that have curtailed antiserotonergic treatment strategies in cardiovascular medicine in the past. Here we report that during AAI serotonin accumulates in the airways after release from platelets in human and animal models. Furthermore, we show that DCs require activation by serotonin to orchestrate the full response in models of AAI.

We demonstrate for the first time that in patients with asthma levels of 5-HT in BALF are increased compared with healthy control subjects. Moreover, allergen exposure of either sensitized animals or patients with allergic asthma leads to further accumulation of 5-HT in BALF. Interestingly, in BALF of patients with asthma, 5-HT concentration of up to 50 ng/ml could be detected, which is approximately 10-fold higher than 5-HT levels measured in platelet-free plasma, suggesting active release of 5-HT into the alveolar space after allergen exposure (21, 38). This is supported by the finding that allergen provocation was associated with a decrease in serum 5-HT in individuals with asthma suggesting an additional translocation from platelet stores into the lung during this acute inflammatory event.

Although the magnitude of this result was surprising, the observation that bronchoconstriction correlates with 5-HT release is in accordance with the observation that free plasma 5-HT concentrations are higher in symptomatic patients, reflecting uncontrolled disease and inflammation, as compared with symptoms-free patients (21). This is in accordance with the present finding that increased 5-HT levels could also be observed in BALF from stable asthma when compared with healthy control patients, suggesting a persistent inflammatory response in the airway.

In other lung and vascular diseases, release of 5-HT has been attributed to platelets. In pulmonary hypertension 5-HT increased pulmonary artery smooth muscle cell contraction and hyperplasia in a TPH1-, 5-HT–specific transporter-, and serotonylation-dependent manner (38–42). In extension from these well-established effects of 5-HT on vascular tone, our data now indicate that large amounts of 5-HT are mobilized locally during AAI. We therefore speculate that this increase in 5-HT might contribute to airway smooth muscle hypertrophy and airway tone in asthma.

Examining TPH1$^{-/-}$ mice we next established that OVA-induced AAI critically depends on serotonin synthesized from nonneuronal cells. We confirmed this result by pharmacologic TPH1 inhibition with PCPA. Both sets of experiments showed a reduction in BALF eosinophils by approximately 50%, together with a reduced cytokine release, a reduction in peribronchiolar inflammation as assessed by histology, and a significant attenuation of bronchial hyperresponsiveness to methacholine. We confirmed the involvement of TPH1 in AAI in a more relevant model of experimental asthma using intratracheal exposure to house dust mite extract, which also demonstrated that TPH1 is pivotal for the development of AAI (*see* Figure E1). These data underline the importance of nonneuronally derived serotonin for AAI, suggesting that it might

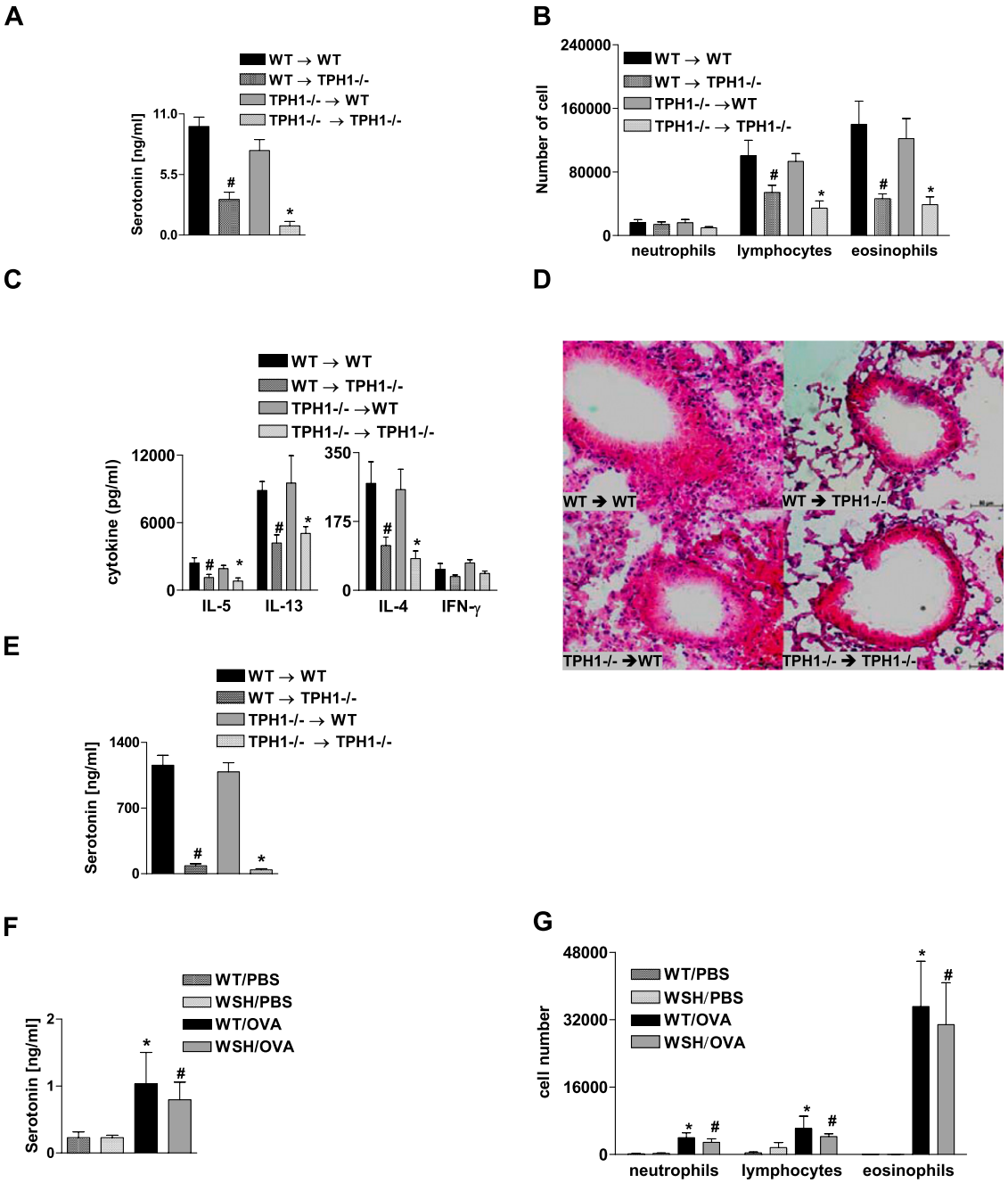

Figure 3. The nonhematopoietic system is the major source of serotonin (5-HT) in allergic airway inflammation. (*A–D*) The different bone marrow (BM) chimera animals were sensitized and challenged to ovalbumin (OVA) as described in the METHODS section. 5-HT level (*A*) and cell differentiation (*B*) in the bronchoalveolar lavage fluid were analyzed by ELISA or flow cytometry. (*C*) Cytokine production in mediastinal lymph node cells restimulated *in vitro* for 4 days with OVA. (*D*) Hematoxylin and eosin staining of lung sections. (*E*) Serum levels of 5-HT were addressed as described in the METHODS section. Data are shown as mean ± SEM; n = 6–8 mice in each group. One experiment out of three independent is shown. $^{*}P < 0.05$, tryptophan hydroxylase (TPH)-$1^{-/-}$ in TPH$1^{-/-}$ versus wild-type (WT) in WT; $^{\#}P < 0.05$, WT in TPH$1^{-/-}$ WT versus WT in WT. (*F* and *G*) Mast cells are not the source of 5-HT in allergic airway inflammation. Mast cell–deficient (KitWsh/KitWsh) and WT (Kit+/Kit+) animals were sensitized and challenged to OVA. (*F* and *G*) 5-HT level and cell differentiation in the bronchoalveolar lavage fluid was addressed by ELISA and flow cytometry. Data are shown as mean ± SEM; n = 6–8 mice in each group. $^{*}P < 0.01$, WT OVA sensitized and OVA challenged versus WT sham sensitized and OVA challenged; $^{\#}P < 0.01$, WSH OVA sensitized and OVA challenged versus WSH sham sensitized and OVA challenged. One experiment out of three independent is shown.

be a target for antiserotonergic treatment strategies to reduce some of the pathophysiologic features associated with asthma.

To develop therapeutic strategies that do not interfere with the multitude of other 5-HT effects it is crucial to identify the source from which serotonin is being released. For this purpose BM transplantation not only confirmed the importance of TPH1-derived serotonin for AAI, but also ruled out hematopoietic TPH1 as the source of serotonin secretion. Monocytes and T cells express TPH1 (16, 34, 35); however, in sensitized TPH$1^{-/-}$ recipient mice, which were reconstituted with WT BM, no increase in 5-HT levels was detected in BALF after allergen challenge. These results support the hypothesis that 5-HT is mainly synthesized by structural cells, such as EC, which produce most nonneuronal serotonin in a TPH1-dependent manner and release 5-HT into

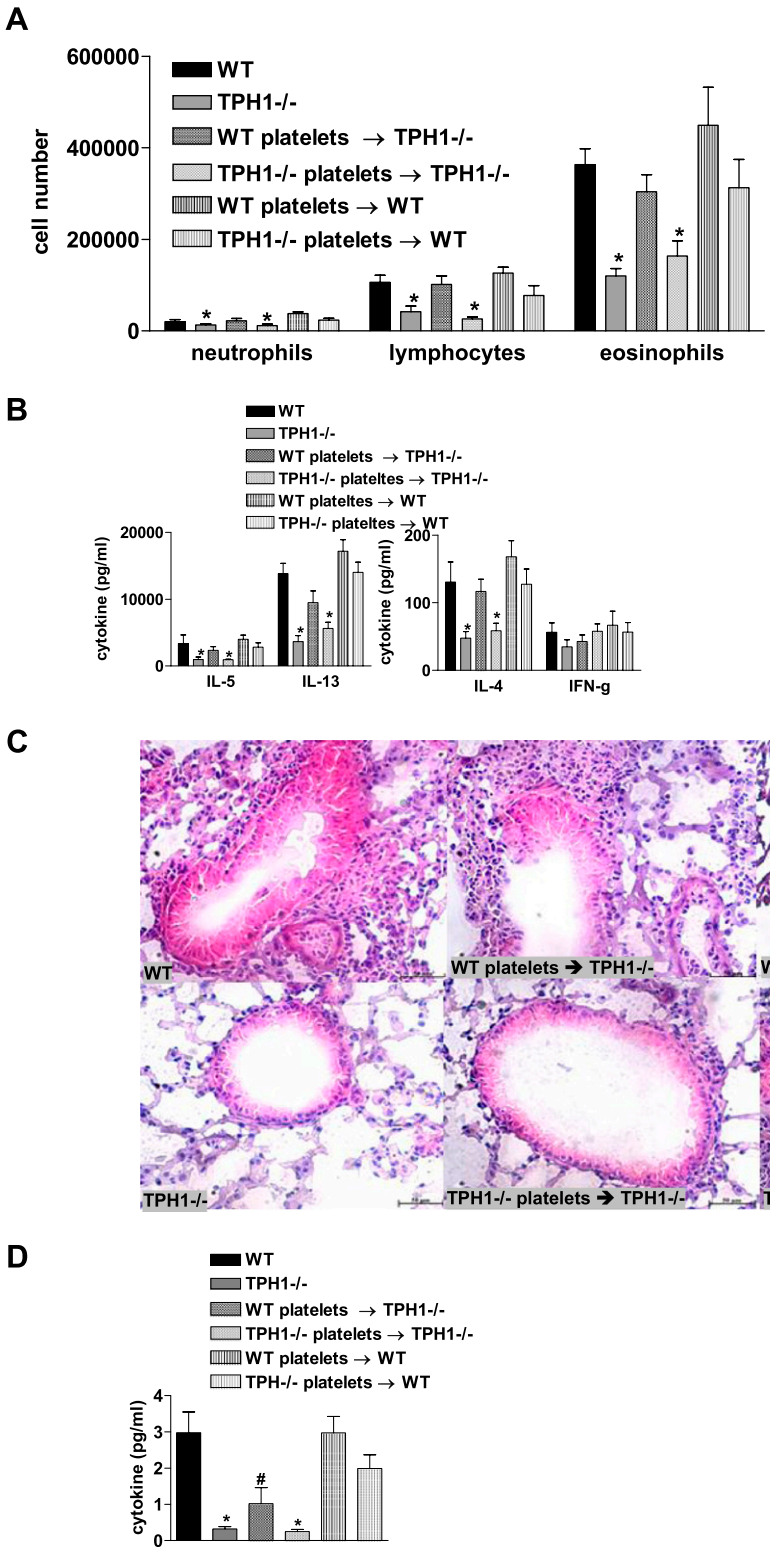

Figure 4. Platelets-released serotonin (5-HT) drives allergic airway inflammation. Wild-type (WT) and tryptophan hydroxylase (TPH)-1$^{-/-}$ mice were sensitized and challenged to ovalbumin (OVA). Some of the animals received intravenous injection of purified WT or TPH1$^{-/-}$ platelets on Days 0, 5, 10, 15, and 20 as described in the METHODS section. Bronchoalveolar lavage fluid cell differentiation (*A*) and 5-HT level (*B*) were analyzed by flow cytometry or ELISA. (*C*) Cytokine production in mediastinal lymph node cells restimulated *in vitro* for 4 days with OVA. (*D*) Hematoxylin-eosin staining of lung sections. Data are mean ± SEM; n = 6–8 mice in each group. *$P < 0.01$. One experiment out of three independent is shown.

blood, where it is stored in platelets and released locally during allergic reactions.

Another potential source of 5-HT could be mast cells, which also express TPH1. Mast cells are derived from BM precursors but then become resident cells, which are also found in the airways. It was unclear whether after irradiation and reconstitution with BM, all mast cells were of the same genotype (17). To assess the role of mast cells as a source of 5-HT in our model, we also investigated mast cell–deficient mice. The role of mast cells for the development of AAI is still controversial and the necessity for the presence of mast cells seems to depend on the model used (43). In accordance with previous findings in the OVA-alum model, we were unable to detect a significant effect of genetic mast cell depletion on the extent of AAI (44, 45). In addition, BALF 5-HT levels were not different in mast cell–deficient and WT animals, suggesting that mast cells are not a major source of 5-HT in this model of AAI.

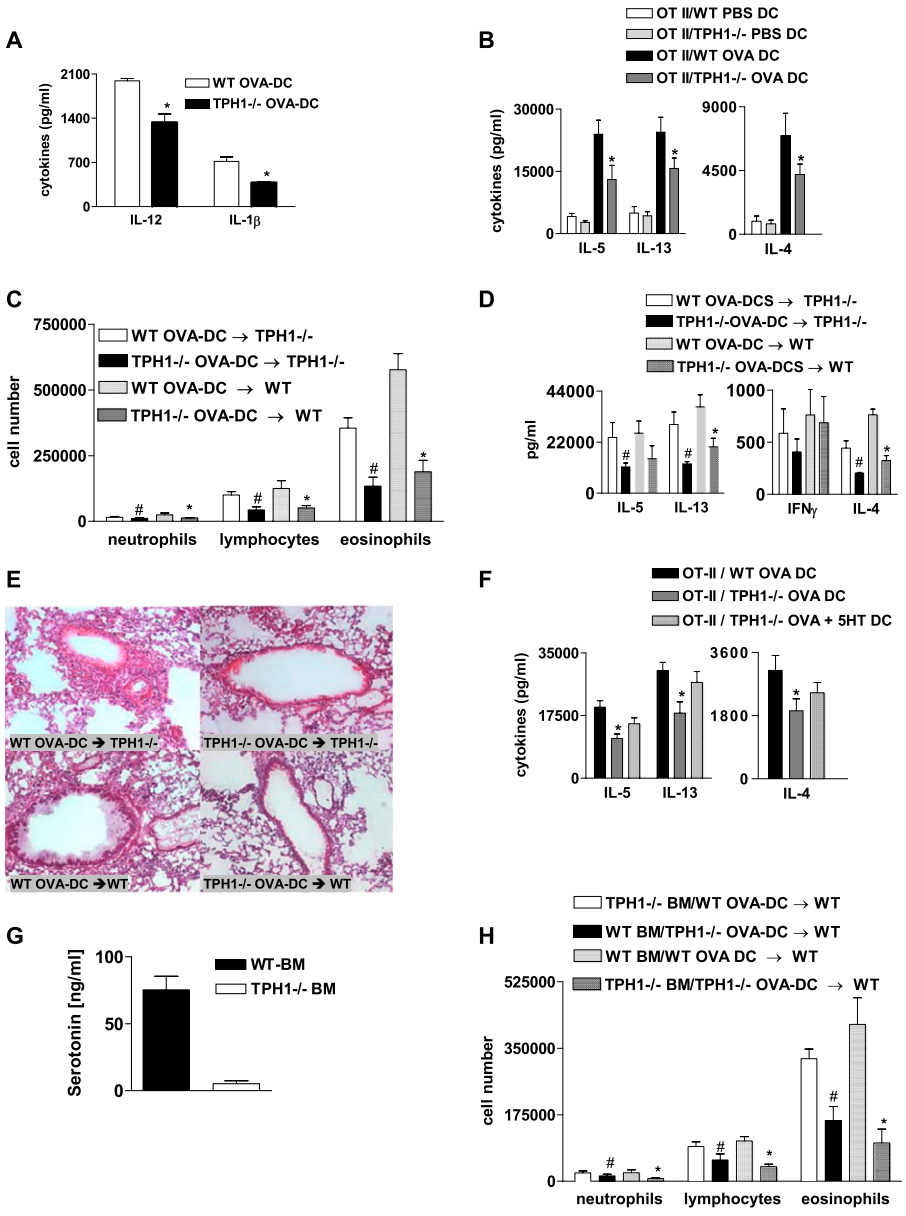

Figure 5. Involvement of functional tryptophan hydroxylase (TPH)-1/endogenous serotonin (5-HT) in cytokine and T-cell priming capacity of dendritic cells (DCs) *in vitro* and *in vivo*. (*A*) Bone marrow dendritic cells (BMDC) from wild-type (WT) and TPH1$^{-/-}$ were pulsed overnight in ovalbumin (OVA) and the levels of tumor necrosis factor-α and IL-1β in the supernatant were detected by ELISA. Data are mean ± SEM; n = 6–8 mice in each group; *$P < 0.01$. One experiment out of three independent is shown. (*B*) On Day 2 mice were injected intravenously with OVA-specific naive T cells from OT II mice. On Day 0, mice were instilled intratracheally with unpulsed or OVA-plused BMDC generated from WT or TPH1$^{-/-}$ mice. Four days later, mediastinal lymph node cells were collected and cultured in 96-well plates for 4 days. The presence of IL-4, IL-5, and IL-13 in the supernatants was analyzed by ELISA. Data are mean ± SEM, n = 6 animals per group *$P < 0.05$. One experiment out of three independent is shown. (*C–E*) On Day 0, OVA-pulsed DC from WT or TPH1$^{-/-}$ mice were intratracheally injected in WT or TPH1$^{-/-}$ mice. From Day 10 to Day 13, all mice were exposed to OVA aerosols. (*C*) Bronchoalveolar lavage fluid was analyzed by flow cytometry. (*D*) Cytokine production in mediastinal lymph node cells restimulated *in vitro* for 4 days with OVA. (*E*) Hematoxylin-eosin staining of lung sections. Data are mean ± SEM. n = 6–8 mice in each group. One experiment out of three independent is shown. *$P < 0.01$ TPH1$^{-/-}$ OVA-DC in WT versus WT-OVA-DC in WT; $^{\#}P < 0.01$ TPH1$^{-/-}$ OVA DC in TPH1$^{-/-}$ versus WT-OVA-DC in TPH1$^{-/-}$. (*F*) On Day 2 mice were injected intravenously with OVA-specific naive T cells from OT II mice. On Day 0, mice were instilled intratracheally with vehicle–OVA-pulsed or 5-HT–OVA-pulsed BMDC generated from TPH1$^{-/-}$ and WT mice, respectively. Four days later, LN cells were collected and cultured in 96-well plates for 4 days. The presence of IL-4, IL-5, and IL-13 in the supernatants was analyzed by ELISA. Data are mean ± SEM, n = 6 animals per group. *$P < 0.05$. One experiment out of three independent is shown. (*G*) 5-HT levels in freshly isolated BM from WT and TPH1$^{-/-}$ mice were detected by ELISA. Data are mean ± SEM. n = 4 mice in each group. (*H*) On Day 0 BMDC generated from the different BM chimera were intratracheally injected in WT mice. From Day 10 to Day 13, all mice were exposed to OVA aerosols. Bronchoalveolar lavage fluid cell differentiation was analyzed by flow cytometry. Data are mean ± SEM. n = 5–8 mice in each group. One experiment out of two independent is shown. *$P < 0.01$, TPH1$^{-/-}$ BM/TPH1$^{-/-}$ OVA-DC in WT versus WT BM/WT-OVA-DC in WT; $^{\#}P < 0.01$, TPH1$^{-/-}$ BM/WT OVA DC in WT versus WT BM/TPH1$^{-/-}$ OVA-DC in WT.

Together, these results imply the following sequence in the synthesis and delivery of peripheral 5-HT: synthesis by intestinal EC cells via TPH1, storage and transport by circulating platelets, and targeted release at the site of inflammation. Indeed, this mechanism was confirmed by platelet transfusion experiments. Platelets from WT mice could restore the full allergic airway response in TPH1$^{-/-}$ recipient mice, whereas platelets from TPH1$^{-/-}$ mice could not. We conclude that 5-HT released from platelets are associated with the recruitment of inflammatory cells into the inflamed airways after allergen exposure. This is in line with previous studies, which showed that 5-HT is an important amplifier of *in vitro* migration of eosinophils (6). It has been well established that platelet activation is a central feature of AAI (46, 47). Platelets were shown to extravasate actively into the lung and to promote the recruitment of eosinophils and lymphocytes in murine models of allergic inflammation (48–50). Recently, binding of activated platelets to eosinophils was found in patients with asthma (51). Complexes of activated platelets expressing P-selectin on their surface binding to β_1-integrin–activated eosinophils seemed to migrate into the lungs of patients with severe asthma and during exacerbations. Together, these data demonstrate that serotonin released locally by activated platelets (likely after extravasation) may be a crucial amplifier of AAI, influencing inflammatory cell movement and function.

We hypothesized that platelet serotonin may promote AAI by activating DCs, because DCs are crucial for initiating and maintaining AAI and recently, serotonin has been shown to be an important modifier of DC function (10, 12, 29, 31, 52, 53). To initiate AAI after acquiring the allergen, allergen-loaded DCs have to follow three steps: they must (*1*) emigrate from the lung into the draining MLN, (*2*) mature into fully potent DCs, and (*3*) deliver activation and coactivation signals to induce allergen-specific Th2

cell differentiation. With respect to the initial step, no significant difference in the migratory capacity of OVA fluorescein isothiocyanate–carrying WT and TPH1$^{-/-}$ DCs from the lung to the draining lymph could be observed (data not shown). However, OVA-pulsed BMDC from TPH1$^{-/-}$ mice showed a lower expression of the costimulatory molecules CD40, CD80, CD86, and CD83 compared with WT-OVA DCs. It has previously been shown that stimulation of T cells by DCs via the CD80-CD86 complex is essential for the differentiation of Th2 cells from naive T cells (31, 33, 54) and administration of soluble CD83 has been reported to inhibit DC-mediated T-cell stimulation and allergen-induced Th2 differentation (55–58), thus suggesting a "defective" T-cell priming capacity of OVA-pulsed TPH1$^{-/-}$ DC. Supporting this concept, OTII Th2 cell differentiation in the MLN after intratracheal injection of OVA-pulsed TPH1$^{-/-}$ DCs was significantly reduced as compared with OVA-pulsed WT DCs. To further elucidate a potential involvement of TPH1 on DCs in T-cell priming in AAI, we used a model in which AAI is induced by adoptive transfer of OVA-pulsed DCs to the lungs of naive animals, followed by OVA challenge. Of note, WT animals receiving OVA-pulsed TPH1$^{-/-}$ DCs showed a significant reduction of cardinal features of AAI as compared with animals that had received OVA-pulsed DCs from WT mice. However, there was only trend of reduced BALF eosinophil numbers in TPH1$^{-/-}$ animals receiving OVA-pulsed DCs from WT mice as compared with WT recipient animals. This decrease in eosinophils in the latter case could be explained by the lack of 5-HT–induced migration of eosinophils (6). The precise mechanisms leading to the reduced capacity of TPH1$^{-/-}$ DCs to induce airway inflammation is unclear but could be caused by either a reduced costimulatory expression by DCs or by an altered cytokine production after OVA exposure. BMDCs from TPH1$^{-/-}$ mice produced significantly lower levels of IL-12 and IL-1β. Interestingly, mature IL-1β has previously been reported to contribute to DC-driven Th2 cell activation and proliferation (28, 59).

These data suggest that endogenous 5-HT, either delivered in BM or produced by DCs themselves, is essential to achieve full Th2 priming capacity. The latter was unlikely because in contrast to freshly isolated BM cells, we could not detect sufficient expression of TPH1 mRNA in BMDCs and in primary isolated spleen DCs (*see* Figure E2; and data not shown). Thus, we hypothesized that 5-HT delivered to BM cells contributes to the maturation and function BMDC *in vitro* and *in vivo*. In line with that, when TPH1$^{-/-}$ DCs were grown in 5-HT–supplemented medium they regained their capacity to prime Th2 immunity *in vivo*. Furthermore, high amounts of 5-HT could be detected in freshly isolated BM cells from WT but not from TPH1$^{-/-}$ mice, supporting the hypothesis that BM was "loaded" with 5-HT *in vivo*. Finally, OVA-pulsed DCs generated from WT animals reconstituted with TPH1$^{-/-}$ BM showed a comparable maturation and Th2 priming capacity *in vivo* compared with those of irradiated WT animals reconstituted with WT BM. Of note, OVA-pulsed DCs from irradiated TPH1$^{-/-}$ recipients reconstituted with WT BM showed an impaired maturation and Th2 priming capacity *in vivo*.

Together, these results suggest that serotonin delivered to BM *in vivo* is required for full maturation and Th2 priming capacity of DCs in AAI. These data are consistent with a recent report about the importance of endogenous 5-HT in DC activation during gut inflammation (60). Whether platelets are also the source of 5-HT for BM cells still needs to be elucidated in further studies.

In summary, we provide evidence that serotonin is released locally by platelets during AAI to promote inflammatory cell recruitment, cytokine and mucus production, and bronchoconstriction. Furthermore, endogenous 5-HT delivered to the BM *in vivo* contributes to the maturation and T-cell priming capacity of BMDCs. Inhibiting the effects of platelet-derived serotonin in the lungs of patients with asthma might be a new therapeutic option that requires further investigation.

Author disclosures are available with the text of this article at www.atsjournals.org.

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