

A tryptophan metabolite, kynurenine, promotes mast cell activation through aryl hydrocarbon receptor

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Abstract

Background: Tryptophan metabolites have been suggested to play a role in immune modulation, wherein those have recently been shown to be endogenous ligands of aryl hydrocarbon receptor (AhR; a unique cellular chemical sensor). However, the involvement of tryptophan metabolites and AhR in modulating mast cell function remains to be fully defined. We therefore investigated that the functional impacts of tryptophan metabolites on human and mouse mast cell responses *in vitro* and their functional importance *in vivo*.

Methods: Three tryptophan metabolites, kynurenine (KYN), kynurenic acid (KA) and quinolinic acid (QA), were examined in terms of their effect on IgE-mediated responses in mouse bone marrow-derived mast cells (BMMCs) and in human peripheral blood-derived cultured mast cells (HCMCs) and on *in vivo* anaphylactic responses. For evaluation of AhR involvement, we examined the responses of mast cells from AhR-null or AhR-wild-type mice with the use of a known AhR antagonist, CH223191.

Results: Kynurenine, but not KA and QA, enhanced IgE-mediated responses, including degranulation, LTC₄ release, and IL-13 production in BMMCs through the activation of PLCγ1, Akt, MAPK p38, and the increase of intracellular calcium. KYN also enhanced cutaneous anaphylaxis *in vivo*. These enhancing effects of KYN were not observed in AhR-deficient BMMCs and could be inhibited by CH223191 in BMMCs. Further, KYN had similar enhancing effects on HCMCs, which were inhibited by CH223191.

Conclusion: The AhR-KYN axis is potentially important in modulating mast cell responses and represents an example of AhR's critical involvement in the regulation of allergic responses.

Tryptophan, an essential amino acid, and its metabolites are suggested to be involved in the regulation of the immune system (1–10). Tryptophan is metabolized mainly through two different biosynthetic pathways: the generation of the neurotransmitter serotonin and the formation of kynurenine

(KYN) derivatives (9, 10). This latter pathway is initiated by cleavage of the indole ring by two different rate-limiting enzymes: indoleamine 2,3-dioxygenase (IDO) found in many immune cells, such as dendritic cells and macrophages, and tryptophan 2,3-dioxygenase (TDO) located in not only liver and neuron, but also some tumor cells such as malignant glioma cells (7, 9, 11). Metabolites generated from this IDO/TDO pathway have been considered as potential ligands for aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, originally discovered as a receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (12).

Aryl hydrocarbon receptor has also been recognized as a receptor for many of the common environmental

Abbreviations

AhR, aryl hydrocarbon receptor; BMMCs, bone marrow-derived mast cells; FICZ, 6-formylindolo[3,2-b]carbazole; HCMCs, human peripheral blood-derived cultured mast cells; Hex, β-hexosaminidase; IDO, indoleamine 2,3-dioxygenase; KA, kynurenic acid; KYN, kynurenine; OVA, ovalbumin; QA, quinolinic acid.

contaminants, dietary components, and heme derivatives (12). Also, a considerable amount of evidence suggests that AhR signaling plays a role in the modulation of the immune system. Recent discovery of the influence of AhR on the balance of Tregs and Th17 cells and its impact on dendritic cell function highlights the potential importance of the AhR ligand axis in immune regulation (13–15). Further, it has been suggested that AhR signaling is also involved in multiple aspects of normal physiology of xenobiotic metabolism, such as modulation of host immunity and maintaining cellular homeostasis (2–6, 16, 17).

Mast cells are known to be critical in the regulation of allergic responses and mucosal immunity (18, 19). Mast cells can be activated by a multitude of stimuli resulting in the release of inflammatory mediators and cytokines, contributing to various pathophysiological events in acute and chronic inflammation (18–20). We have recently reported that AhR is critical in controlling mast cell differentiation, growth, and function (21). Considering the strategic location of mast cells at the site of tissue mucosa where exposure of tryptophan and its metabolites may occur, we therefore hypothesized that there may exist a potential regulatory effect of tryptophan metabolites in controlling the mast cell responses.

In this study, we have investigated the effects of three tryptophan metabolites on IgE-mediated responses in mouse and human mast cells and showed that KYN, but not kynurenic acid (KA) and quinolinic acid (QA), was able to promote mast cell responses through AhR.

Methods

All methods and experimental procedures used in this study are described in detail in Supporting Information, including mice, chemical reagents, generation of mouse bone marrow-derived cultured mast cells (BMMCs) and human peripheral blood-derived cultured mast cells (HCMCs), assays for

β -hexosaminidase release as mast cell degranulation, LTC₄ release and IL-13 production in culture supernatant, *in vivo* passive cutaneous anaphylaxis, intracellular calcium measurement, immunoblotting and immunoprecipitation, and statistical analysis. All experiments were approved by the Animal Care and Use Committee at National Health Research Institutes, Taiwan.

Results

KYN potentiates IgE-mediated mast cell activation

To explore the potential functional impacts of tryptophan metabolites in regulating mast cell function, mouse BMMCs, which constitutively expressed AhR and responded to its ligands (21, 22), were used as a model. To examine the effects of KYN, KA, and QA on Fc ϵ RI-mediated mast cell degranulation, BMMCs were sensitized with ovalbumin (OVA)-specific IgE mAbs in the presence or absence of these tryptophan metabolites for varying time points, followed by cross-linkage of surface-bound IgE with antigen, OVA, for 30 min. The results showed that while tryptophan metabolites alone were unable to induce release of β -hexosaminidase, a preformed granule mediator, significantly enhanced levels of degranulation were found in mast cells pretreated with varying dosages of KYN (Fig. 1A), according to the commonly used dosing protocol (21), but not by KA nor QA for up to 1 mM (Fig. S1). In addition, no enhancing effect was noted when the cells were pretreated with KYN for 10 min just before OVA challenge (data not shown). The need for pretreatment of the cells with KYN for observing the effect is consistent with previous reports in other experimental systems using different AhR ligands (21, 23). In addition, the enhanced LTC₄ secretion (Fig. 1B) and IL-13 production (Fig. 1C) by KYN were also seen in stimulated mast cells. Furthermore, there were neither additive nor synergistic

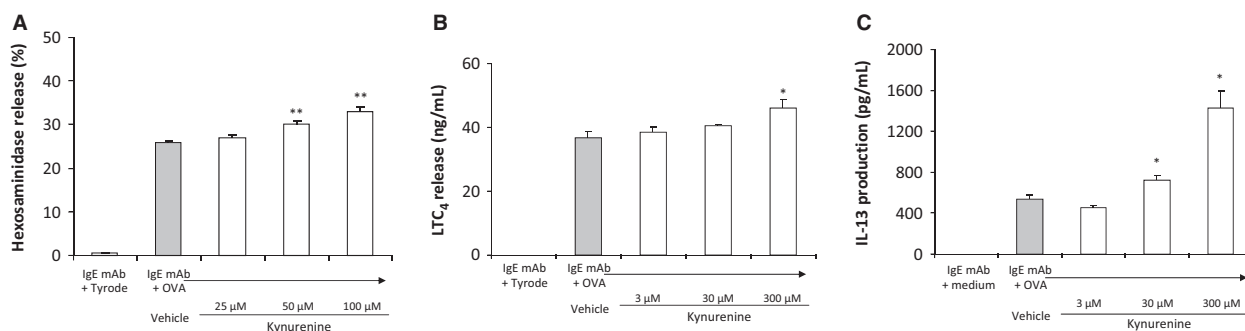


Figure 1 Kynurenine (KYN) potentiates IgE-mediated mast cell activation *in vitro*. (A) KYN enhances IgE-mediated degranulation. Bone marrow-derived mast cells (BMMCs) from C57BL/6 mice were sensitized with anti-ovalbumin (OVA) IgE (E-C1) $\pm$ 25, 50, 100 μ M KYN or vehicle as control for 16 h, then stimulated with 0.5 μ g/ml OVA for 30 min. Degranulation was monitored by the release of β -hexosaminidase. ** P < 0.01 vs vehicle. Data are representative of three independent experiments. (B) KYN enhances

IgE-mediated LTC₄ release. BMMCs were sensitized with E-C1 $\pm$ 3, 30, 300 μ M KYN or vehicle for 16 h, then washed, and stimulated with 0.5 μ g/ml OVA for 30 min. * P < 0.05 vs vehicle. Data are representative of three independent experiments. (C) KYN enhances IgE-mediated IL-13 production. BMMCs were sensitized with E-C1 $\pm$ 3, 30, 300 μ M KYN or vehicle for 16 h, then washed, and stimulated with 0.5 μ g/ml OVA for 6 h. * P < 0.05 vs vehicle. Data are representative of three independent experiments.

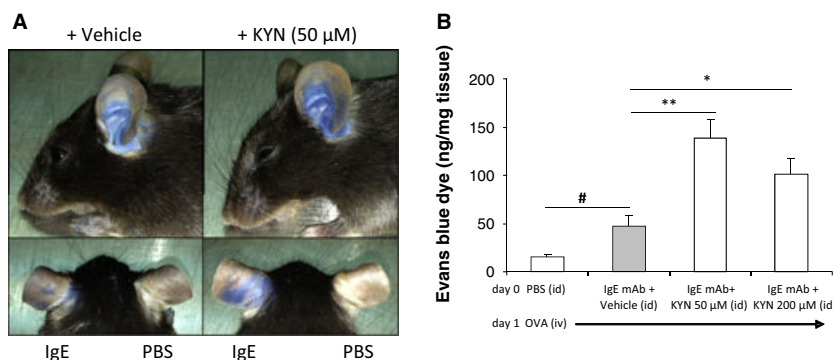


Figure 2 Kynurenine (KYN) potentiates anaphylactic responses *in vivo*. (A) Representative pictures of the reaction sites at 30 min after antigen challenge. (B) KYN enhances PCA reactions. Mice were injected intradermally in the left ear with 200 ng E-C1 in 10 μ l $\pm$ 50, 200 μ M KYN or vehicle as positive control, and in the right ear with 10 μ l PBS as negative control. After 24 h, 0.5 mg

ovalbumin (OVA) was administered intravenously together with Evans blue dye, followed by measurement of the extravasations of Evans blue dye into the ear. # P < 0.05 vs negative control, * P < 0.05, ** P < 0.01 vs positive control. Data are shown as mean $\pm$ SEM of four mice.

effects observed when mast cells were treated with either a combination of Kyn and KA or QA or a combination of all three metabolites (Fig. S2). Mast cells remained viable in all conditions ($\geq 95\%$, data not shown).

To evaluate whether KYN has a similar effect *in vivo*, a model of passive cutaneous anaphylaxis (PCA) was examined, wherein mice were passively sensitized with PBS or OVA-specific IgE mAb $\pm$ varying doses of KYN. As seen in Fig. 2, a significant enhancement of PCA was noted in mice treated with either 50 μ M or 200 μ M KYN, demonstrating the significant functional impact of KYN on mast cell action *in vivo*.

KYN potentiates signaling events associated with mast cell function

The aggregation of Fc ϵ RI on the surface of mast cells initiates a complex cascade of signaling events, with elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$) as one of the critical common events (24). As seen in Fig. 3A, while, as expected, an immediate as well as sustained $[Ca^{2+}]_i$ increase was observed in mast cells following OVA challenge, a substantial $[Ca^{2+}]_i$ enhancement of both phases was noted in KYN-treated cells. Concomitant with the increased levels of $[Ca^{2+}]_i$, activation of several upstream, and downstream signals was noted in KYN-treated cells upon stimulation with antigen. Notably, KYN-treated cells showed an enhanced level of phosphorylation of PLC γ 1, Akt, and MAPK p38 (Fig. 3B,C), but not JNK and ERK.

AhR is necessary for KYN-mediated enhancing effect on mast cell response

Next, to examine whether AhR is involved in enhancing effects by KYN, BMMCs from AhR-null (AhR KO) mice were used. As seen in Fig. 4A, KYN's enhancing effects on the levels of IgE-mediated intracellular calcium were not

observed in AhR KO mast cells (Fig. 4A). Also, no enhancing effects by KYN or a known AhR ligand, 6-formylindolo [3,2-b]carbazole (FICZ), on the levels of degranulation, LTC $_4$ release, and IL-13 production were found in AhR KO mast cells. It was noted that consistent with previous findings in BMMCs generated from AhR KO mice (21), apparently lower levels of the mast cell response were found in AhR KO mast cells compared with those of WT mast cells.

Therefore, to evaluate whether KYN potentiates IgE-mediated activation through the AhR under appropriate responses, CH223191, an AhR antagonist, was examined in WT BMMCs. CH223191 is a potent and pure AhR antagonist, does not exhibit AhR agonistic activities, and competitively binds to AhR, thereby inhibiting AhR transformation (25, 26). Treatment with CH223191 for 16 h before challenge abolished the enhancing effect by KYN, as well as that by FICZ, on IgE-mediated IL-13 production (Fig. 4C). These results suggest the possible involvement of the AhR-KYN axis in regulating the IgE-mediated murine mast cell responses.

KYN potentiates IgE-mediated responses in human mast cells

To investigate the possibility of similar enhancing effects on human mast cells, HCMCs were used as a model. The results showed that consistent with mouse BMMC results, similar enhancing effects of KYN on degranulation (Fig. 5A) and IL-13 production (Fig. 5B) were found in HCMCs. In addition, increased levels of IL-13 by KYN in HCMCs derived from all subjects tested were abrogated when an AhR antagonist, CH223191, was added (Fig. 5B).

Discussion

In this study, we demonstrated that KYN, a tryptophan metabolite and a potential AhR ligand, significantly

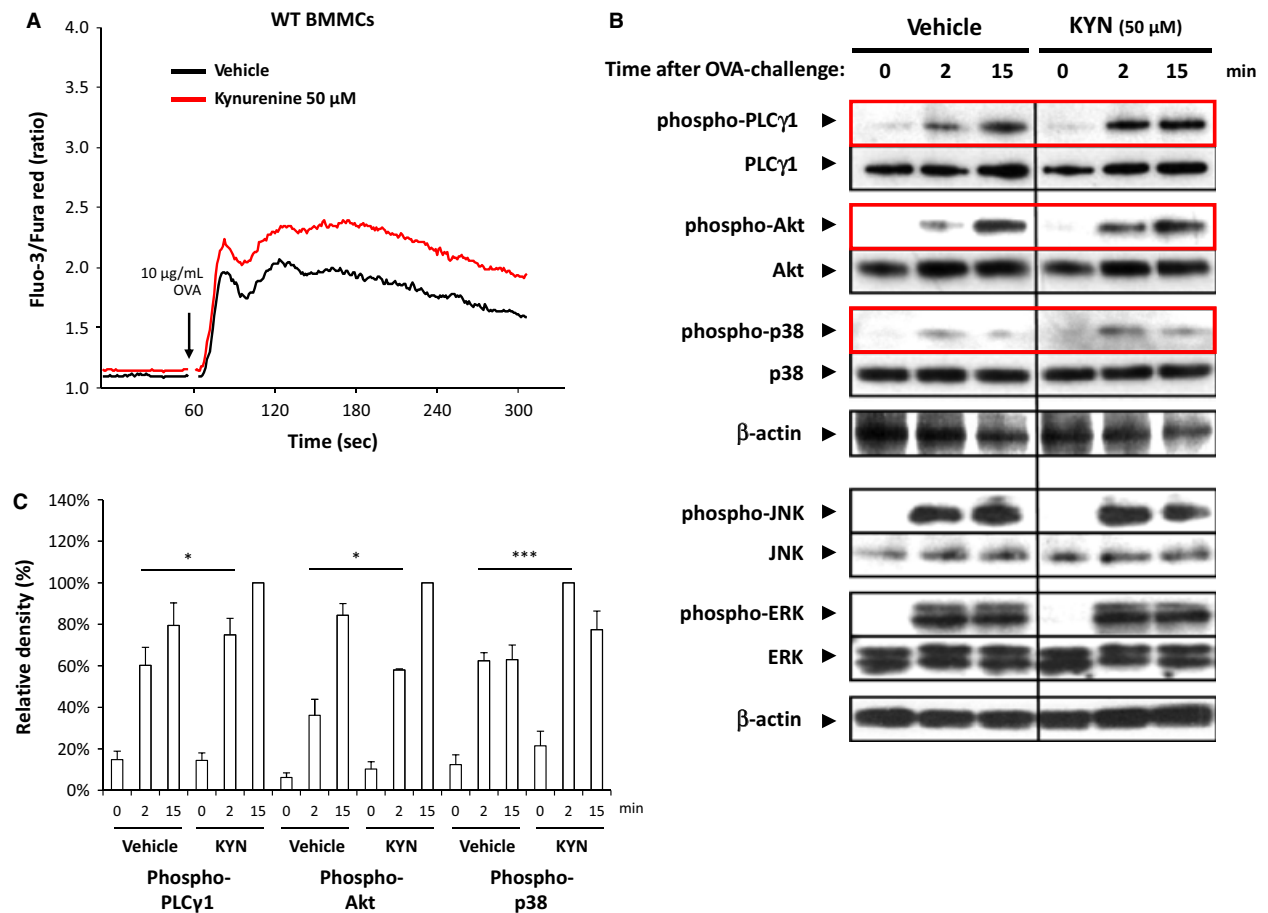


Figure 3 Kynurenine (KYN) potentiates signaling events associated with mast cell function. (A) IgE-mediated Ca^{2+} influx. Bone marrow-derived mast cells (BMMCs) were sensitized with E-C1 $\pm$ 50 μ M KYN or vehicle for 16 h, loaded with the Ca^{2+} indicator Fluo-3-AM, and Fura red-AM, resuspended in Tyrode's buffer containing Ca^{2+} , and stimulated with 10 μ g/mL ovalbumin (OVA) for 4 min. Data are representative of three independent experiments. (B) Immunoblotting analysis of whole-cell lysates. BMMCs were sensitized as above, and then the cells were stimulated with

10 μ g/mL OVA for the indicated time periods. Whole-cell lysates were analyzed by Western blotting. Data are representative of 4–6 independent experiments. (C) Densitometry analysis of the Western blotting of phosphorylated proteins. * P < 0.05, *** P < 0.001; vehicle vs KYN at 2 min after antigen challenge. Data are shown as relative density of phosphorylated proteins corrected by the respective total proteins, and representative of 4–6 independent experiments.

enhanced IgE-mediated mast cell functions *in vitro* and *in vivo*. In the *in vitro* studies, it was shown that KYN promoted the mast cell response, including degranulation, lipid metabolite generation, and IL-13 production, concomitant with the increased activation of the IgE receptor-mediated signaling events, but did not affect the expression level of Fc ϵ RI (Fig. S3). Notably, in KYN-exposed mast cells, enhanced levels of calcium signaling and activated PLC γ 1, Akt and MAPK p38 were found. Importantly, KYN was also shown to be able to enhance cutaneous anaphylaxis *in vivo*. Moreover, KYN's enhancing effects were lost in AhR-null mast cells or when an AhR-selective antagonist was added in the culture of KYN-treated AhR-wild-type mast cells, supporting the importance of AhR in mediating KYN's effect. Furthermore, KYN's enhancing effect on IgE-

mediated responses was also observed in human mast cells, suggesting the potential importance of KYN in regulating human mast cell response.

According to our current understanding, tryptophan degradation along the KYN pathway results in tryptophan depletion as well as the production of a number of metabolites, whose biological activities in mast cells are poorly understood. As tryptophan is converted along the KYN pathway, KYN is the first stable intermediate product. Our studies provide the evidence that KYN plays a role in potentiating IgE-mediated mast cell responses through AhR, suggesting the existence of a potentially novel regulatory pathway in mast cell function when appropriate concentrations of KYN are produced in the allergic and inflammatory micro-environment.

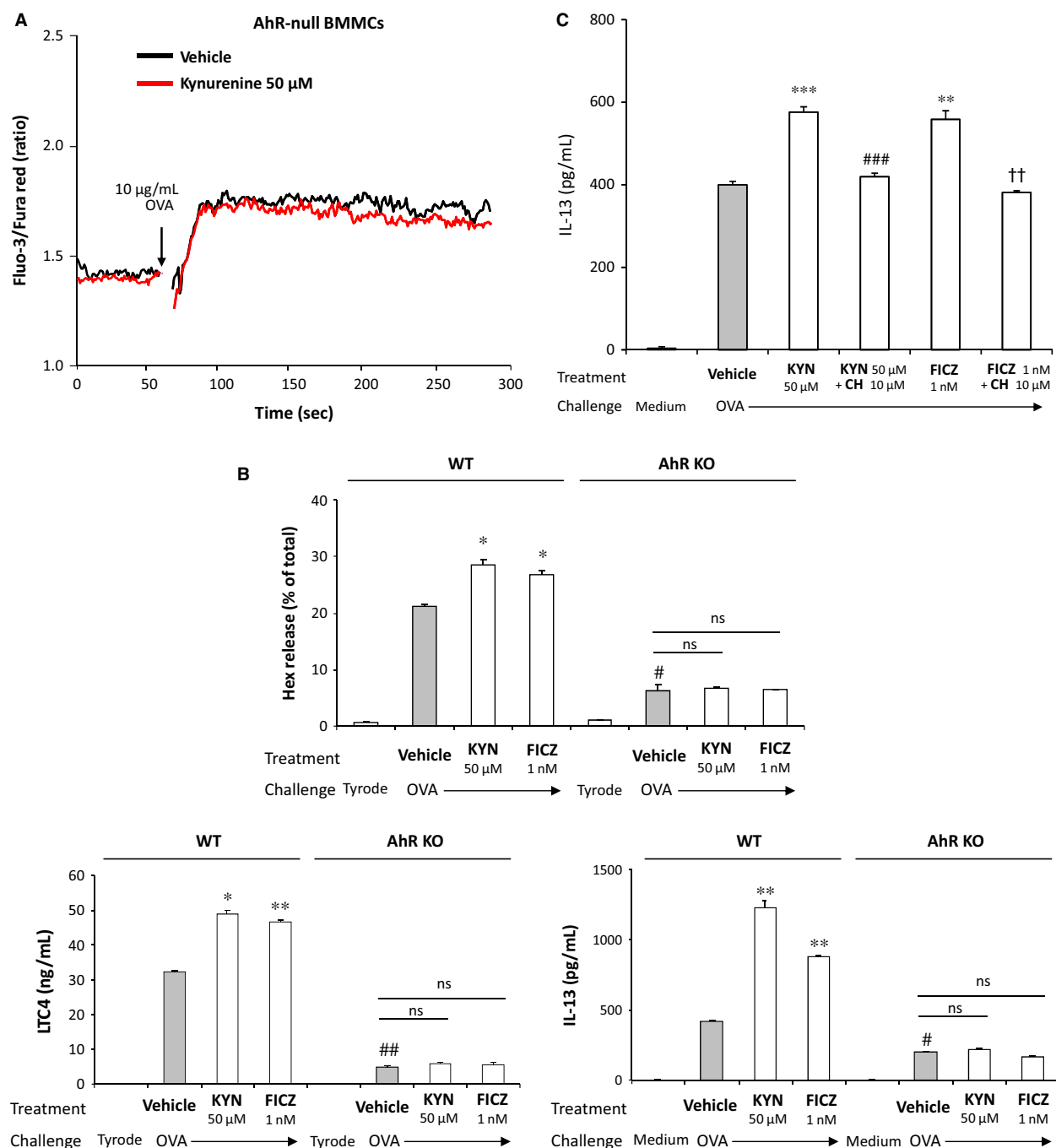


Figure 4 Kynurenine's (KYN) enhancing effect is through aryl hydrocarbon receptor (AhR). (A) Ca^{2+} influx in AhR-null bone marrow-derived mast cells (BMMCs). AhR-null mast cells were sensitized with E-C1 $\pm$ 50 μ M KYN or vehicle for 16 h, loaded with the Ca^{2+} indicators, resuspended in Tyrode's buffer containing Ca^{2+} , and stimulated with 10 μ g/ml ovalbumin (OVA) for 4 min. Data are representative of two independent experiments. (B) IgE-mediated degranulation, LTC_4 release and IL-13 production in WT or AhR-null BMMCs. Mast cells were sensitized with E-C1 for 16 h and stimulated with 0.5 μ g/ml OVA for 30 min for degranulation and LTC_4 , or

6 h for IL-13. * P < 0.05, ** P < 0.01 vs vehicle in WT BMMCs, # P < 0.05, ### P < 0.05 vs vehicle in WT BMMCs. ns; not significant. Data are representative of three independent experiments. (C) Effect of CH223191 (CH) on enhancing effect by KYN (50 μ M) or 6-formylindolo[3,2-b]carbazole (FICZ) (1 nM) of IgE-mediated IL-13 production in WT BMMCs. Cells were sensitized as above in the presence or absence of 10 μ M CH and stimulated with 0.5 μ g/ml OVA for 6 h. ** P < 0.01, *** P < 0.001 vs vehicle, ### P < 0.001 vs KYN, †† P < 0.01 vs 6-formylindolo[3,2-b]carbazole (FICZ). Data are representative of three independent experiments.

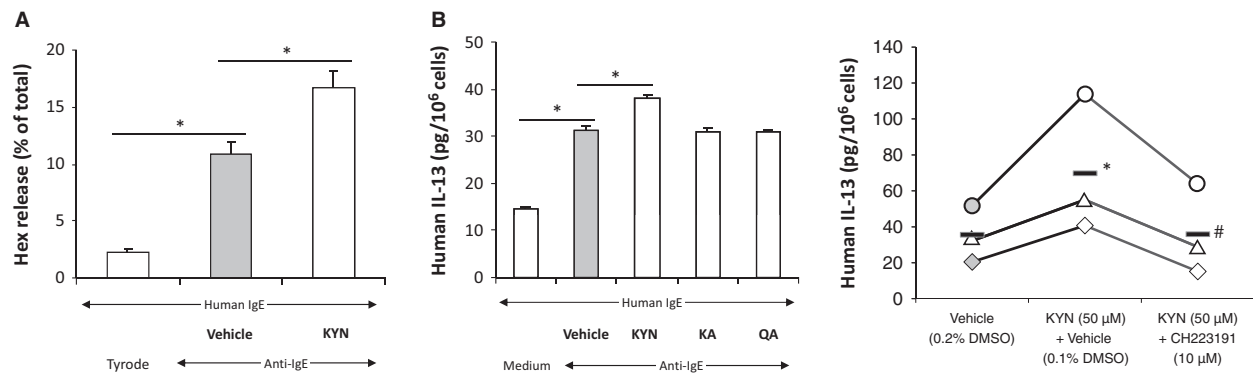


Figure 5 Kynurenine (KYN) potentiates IgE-mediated responses in human mast cells. (A) IgE-mediated degranulation in HCMCs. HCMCs were sensitized with myeloma IgE in the presence of 50 μ M KYN or vehicle as control for 16 h and stimulated with anti-human IgE for 30 min. $*P < 0.05$ vs vehicle. Data are representative of three independent experiments. (B) IgE-mediated IL-13 production in HCMCs. HCMCs were primed with IL-4 for 5 days, then sensitized with myeloma IgE $\pm$ 50 μ M KYN, kynurenic acid

(KA), or quinolinic acid (QA) for 16 h, and stimulated with anti-human IgE for 24 h. $*P < 0.05$ vs vehicle. Data are representative of three independent experiments. (C) Effect of CH223191 on enhancing effect by KYN of IgE-mediated IL-13 production in HCMCs. HCMCs were primed and sensitized as above in the presence or absence of 10 μ M CH223191 and stimulated with anti-human IgE for 24 h. $*P < 0.05$ vs vehicle, $\#P < 0.05$ vs KYN. Data are representative of three independent experiments.

As a corollary, a very recent observational study by Maetoft-Udsen et al. (23) has suggested a potential role of tryptophan metabolites, including KYN and KA, in regulating a rat basophilic leukemia cell line (RBL-2H3 cell), although the dependence of AhR and the underlying mechanisms were not investigated. It was noted in our study that the effective concentration of KYN, but not KA, for showing the enhancing effect on mast cells was around 50 μ M, which has been frequently used in various experimental settings and could be a physiologically feasible concentration in the microenvironment of the site where inflammation occurs (11, 27). Also, the study by Maetoft-Udsen et al. showed that long-term treatment of the RBL cells with KA, but not KYN, enhances degranulation. This inconsistency might be due to the use of a basophilic cell line instead of cultured primary mast cells and the experimental conditions used in the two studies. In addition, pretreatment of growth-factor-independent CLMC/C57.1 mouse mast cells for 1 h with 1 μ M of KA enhanced, while pretreatment with 1 μ M of KYN inhibited, IgE-mediated degranulation, although the difference did not reach statistical significance and the underlying mechanism was not known. It is noted that in the present study for generation of BMMCs, the complete culture medium is composed of 30% of WEHI-3 conditioned medium containing tryptophan, growth factors, and 0.15 μ M of KYN (Fig. S4), suggesting that the exposure of submicromolar level of KYN during the development of mast cells might have an influence on their capability for regulating mast cell responses; but our results clearly showed that 1 μ M of KYN was not sufficient in regulating mast cell function. Interestingly, in the study by Maetoft-Udsen et al., KA was suggested to be an AhR ligand, but its stimulatory effect on a known AhR target gene, CYP1A1, was observed only after 24 h of stimulation, while CYP1A1 expression inducible by AhR ligands, including FICZ (28), is

known to occur within 30 min. This suggests the likelihood of an AhR-independent and/or a secondary activating mechanism. Indeed, KA has been shown to be a ligand for GPR35 (29), a G-protein-coupled receptor expressed in a variety of tissues including mast cells, which was significantly upregulated when mast cells were exposed to IgE antibodies (30). Thus, the possibility of a sequential event of crosstalk between AhR and GPR35-KA axis in regulating mast cell functions may exist. KA has also been suggested as a potential ligand for mouse and human AhR, although its affinity for human is 100-fold higher than that for mouse (31). In our experimental conditions, however, 16-h treatment of KA alone did not induce mast cell responses, including degranulation, in either BMMCs or HCMCs. Further detailed studies with a longer term of stimulation protocol may be needed to examine this issue.

Recently, it has been reported that KYN, which was produced during cancer progression and inflammation in the local microenvironment in amounts sufficient for activating the human AhR, suppresses antitumor immune responses and promotes tumor cell survival and motility through the AhR in an autocrine/paracrine fashion (11). KYN-mediated activation of the AhR is not only relevant in the setting of cancer. Exogenous KYN induces generation of FoxP3⁺ Treg cells from naïve T cells in an AhR-dependent manner (2), and in bone marrow-derived dendritic cells and T cells co-culture system, AhR regulates dendritic cell immunogenicity via a KYN-dependent mechanism (3). Moreover, a complex metabolic interplay between IDO and AhR signaling has been suggested (32). It is thought that cells expressing functional IDO or TDO may modulate local immune effector functions by stimulating AhR in innate and adaptive immune cells, such as T cells, Th17, Tregs, and DCs, which reinforces IDO-mediated regulatory

phenotypes in these cells. However, it has been shown that exposure to IDO inhibitors accelerates autoimmune response and potentiates disease severity in various models (33, 34).

Furthermore, a recent report, in the setting of allergen immunotherapy, suggested that KYN down-regulates allergic responses by potentiating tolerance induction in a mouse model of asthma (35). KYN enhanced the efficacy of suboptimal immunotherapy with OVA, resulting in significant reduction of OVA-induced increase in airway eosinophilia, and Th2 cytokine production. Thus, KYN might potentiate immune tolerance during allergic responses, with anti-allergic properties. Therefore, it is likely that KYN might contribute to potentiating both pro-allergic and anti-allergic responses depending on the types of related immune cells in the progression of allergic diseases, being orchestrated by a variety of immune cells including T cells, dendritic cells, and mast cells.

It is noted that the enhancing effect of KYN was found when mast cells were pretreated with KYN 16 h before OVA challenge, which was consistent with our previous report demonstrating the potentiating effect of known AhR ligands (21). This 'priming' effect is AhR dependent and may be mediated through the generation of mediators with functional impact on mast cell activation, although their identity remains to be discovered. Therefore, further investigation including the impacts of the priming mechanism by KYN would be important for better understanding of its regulatory mechanisms.

In summary, we have demonstrated that KYN promotes IgE-mediated responses in mouse and human mast cells, and its enhancing effect by KYN was dependent on AhR signaling. These findings suggest that KYN may play an important role in controlling mast cell responses through AhR, and the subsequent expression of allergic diseases, considering also the fact that tryptophan is an essential amino acid and has been used as a nutritional supplement.

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Author contributions

H.K. conducted experiments characterizing how tryptophan metabolites regulate IgE-mediated responses through AhR in mast cells, analyzed data, and wrote the article; H.-W.C. performed mouse and human mast cell culture; H.-C.T., S.-C.H., S.-J.Y., C.-H.H., and Y.Z. contributed with interpretation of the data; S.-K.H. planned, designed, supervised, coordinated the overall research efforts, and wrote the article.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Kynurenic acid (KA) and quinolinic acid (QA) do not potentiate IgE-mediated mast cell activation *in vitro*.

Figure S2. Combination treatments of kynurenine and kynurenic acid (KYN + KA), quinolinic acid (KYN + QA), or a combination of all three metabolites (KYN + KA + QA) did not affect the enhancing effects by kynurenine on IgE-mediated mast cell activation.

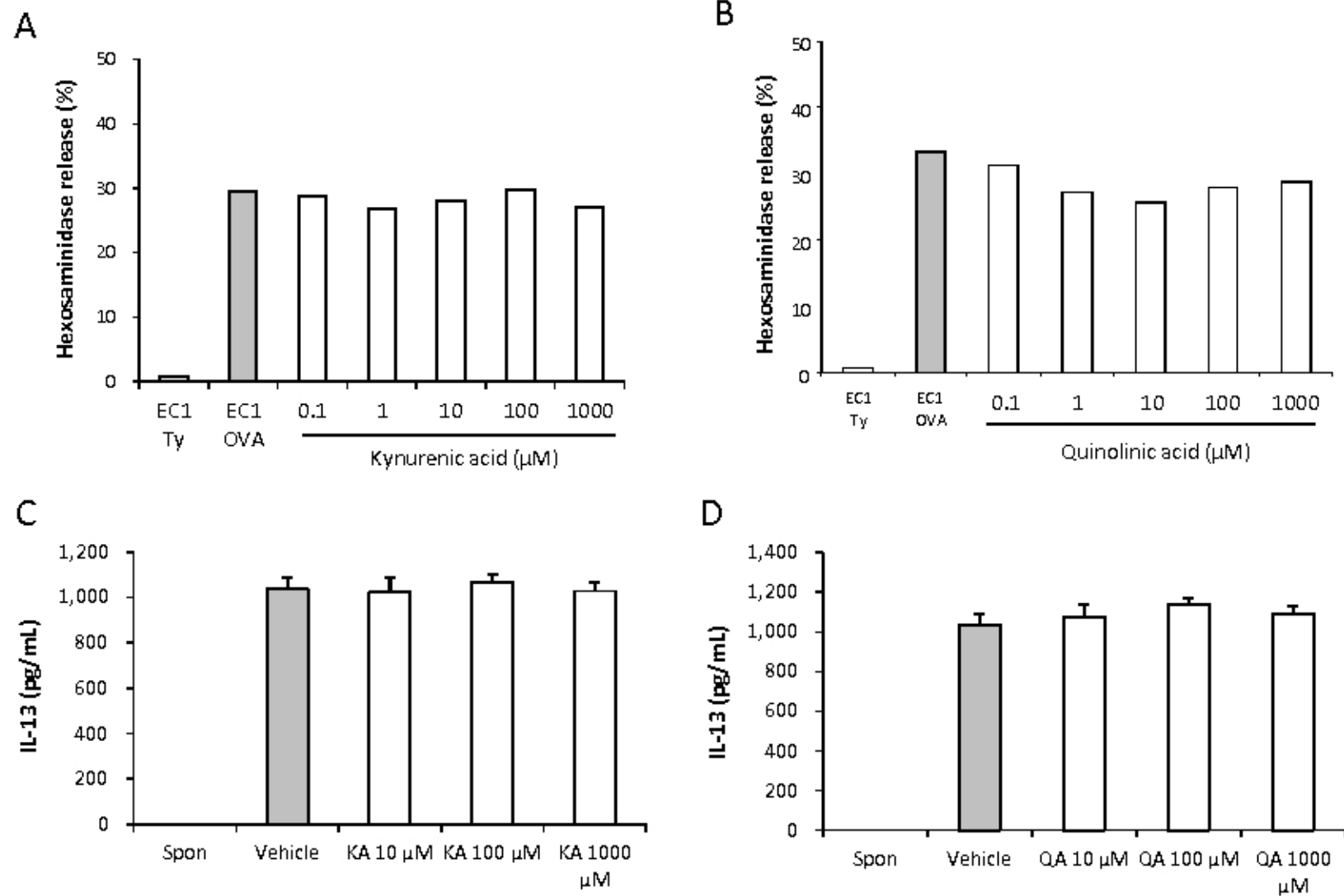
Figure S3. Expression level of FcεRI on BMMCs after treatment of kynurenine.

Figure S4. Kynurenine level in culture medium.

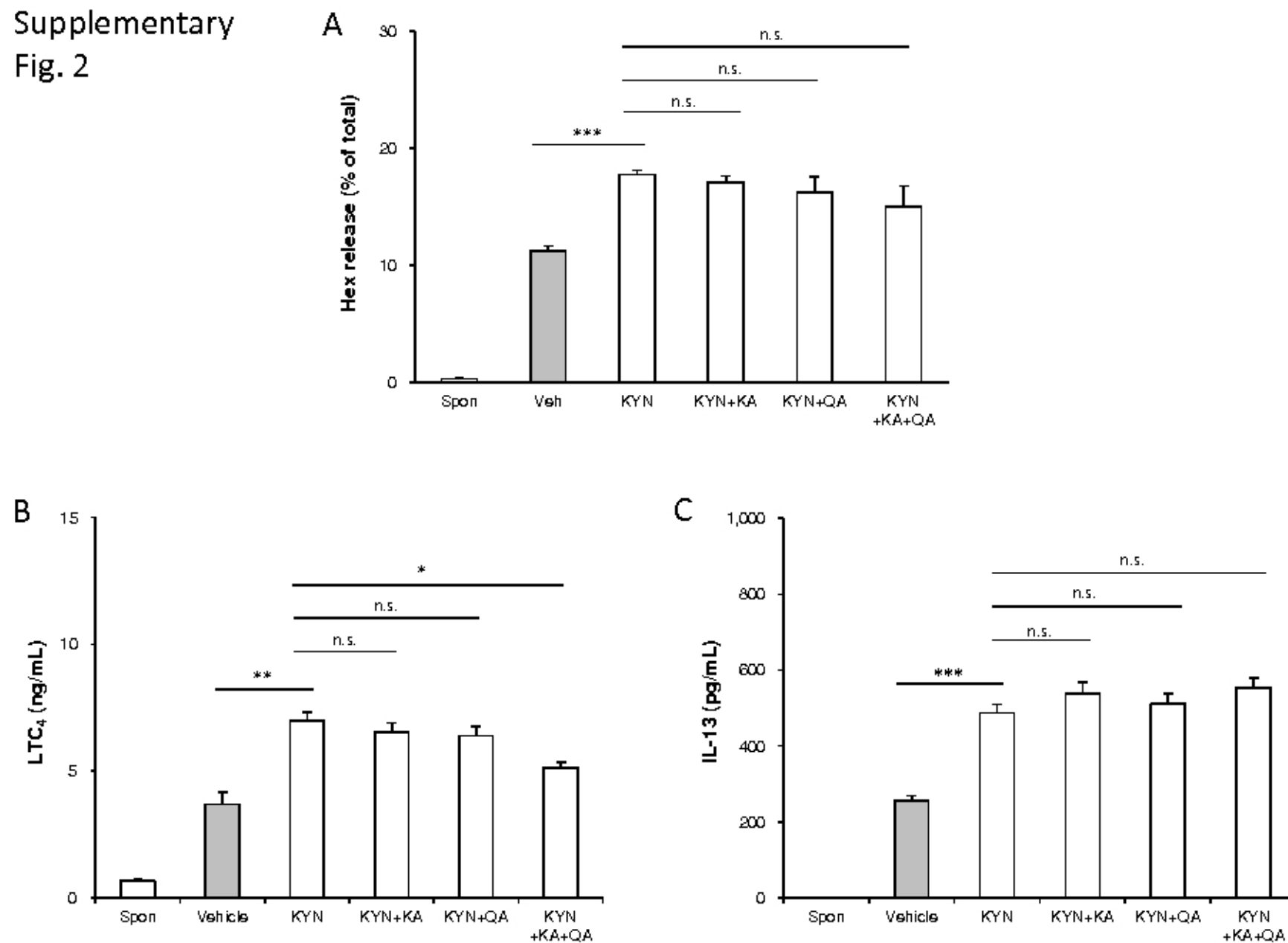
Data S1. Methods.

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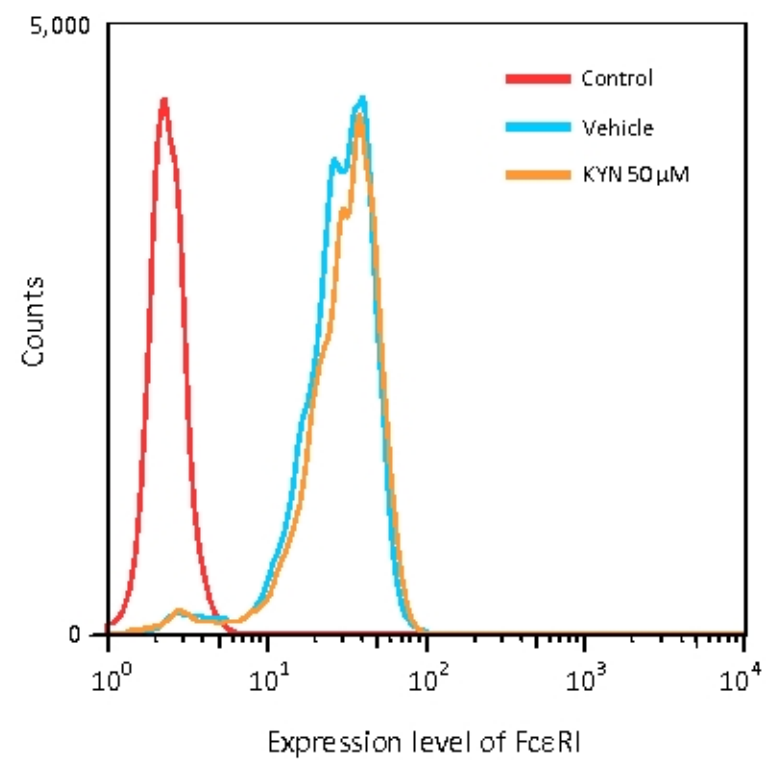
Supplementary
Fig. 1



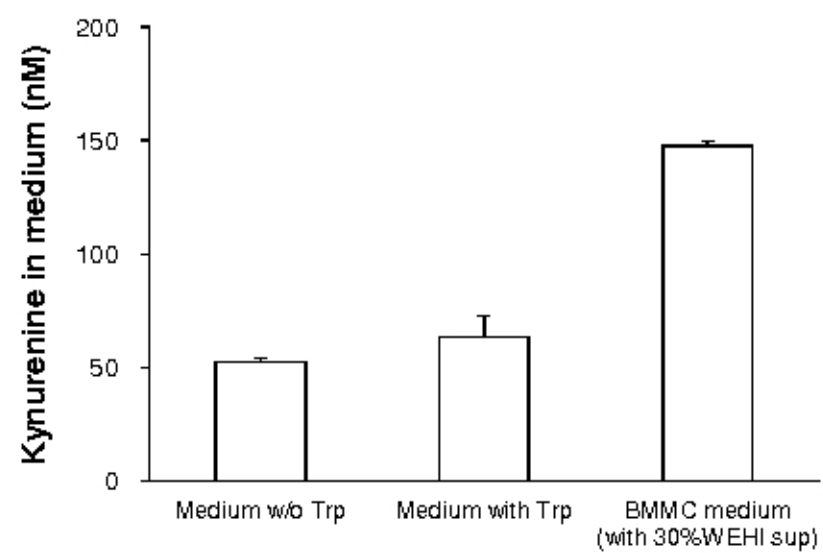
Supplementary
Fig. 2



Supplementary
Fig. 3



Supplementary
Fig. 4



Supporting Information

Methods

Mice. C57BL/6 and AhR-null (WT.129-Ahr^{tm1Bra}/J) mice from the Jackson Laboratory (Bar Harbor, ME) were maintained under specific-pathogen-free conditions, and all experiments were approved by the Animal Care and Use Committee at National Health Research Institutes, Taiwan.

Chemicals and reagents. Kynurenine, kynurenic acid (Sigma-Aldrich), quinolinic acid, CH223191 (Tocris Bioscience), anti-OVA IgE mAb (E-C1, Chondrex Inc.), OVA (EndoGrade[®] Low-endotoxin ovalbumin, < 1 EU/mg, Hyglos GmbH), ionomycin (Invitrogen), 6-Formylindolo(3,2-b)carbazole (FICZ; BioMol-Enzo Life Science), purified human myeloma IgE, and anti-human IgE (Millipore) were used.

Generation of bone marrow-derived cultured mast cells (BMMCs). Mouse bone marrow cells were cultured at a starting density of 5×10^5 cells/mL at 37°C in a humidified atmosphere containing 5% CO₂ in RPMI-1640 medium, supplemented with 2 mM GlutaMAX[™], 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% heat-inactivated FBS (Invitrogen), 50 µM β-2-mercaptoethanol (2-ME), 10 mM HEPES, and 30% WEHI-3-conditioned medium, as a source of IL-3 for 4 to 6 weeks. Mast cell phenotype was confirmed by flow cytometry analysis with Abs specific for *c-Kit* (2B8; eBiosciences) and FcεRI (MAR-1; eBiosciences) and by histochemical staining with acid toluidine blue.

Degranulation in BMMCs. Degranulation was monitored by the release of β-hexosaminidase into the culture supernatants. BMMCs were sensitized with 1 µg/mL anti-OVA IgE with or without the addition of different tryptophan metabolites or equal amount of vehicle at 37°C for 16 h and then washed with Tyrode's buffer. BMMCs (2×10^5 cells/tube, 400 µL final volumes) were stimulated with 0.5 µg/mL OVA for 30 min at 37°C. Reactions were terminated by centrifugation at $500 \times g$ at 4°C for 5 min, and 350 µL of supernatants was collected for β-hexosaminidase assay. Cell lysate of each sample prepared by Tyrode's buffer containing 0.5% Triton X-100 was used to obtain the total β-hexosaminidase content. Degranulation is expressed as a percentage of total β-hexosaminidase activity in each sample.

LTC₄ assays. LTC₄ in supernatant collected on degranulation assays was measured with an EIA kit (Cayman Chemical) according to the manufacturer's instructions.

IL-13 measurement. IgE-sensitized BMMCs (2×10^5 cells) were stimulated with 0.5 or 3 $\mu\text{g/mL}$ OVA for 6 h at 37°C . Reactions were terminated by centrifugation at $500 \times g$ at 4°C for 5 min, and supernatants were collected for IL-13 measurement. Mouse IL-13 in BMMCs supernatants was measured with an ELISA kit (R&D) according to the manufacturer's instructions.

Passive cutaneous anaphylaxis. Mice were anesthetized with isoflurane and sensitized in the left ear by intradermal injection of 200 ng anti-OVA IgE in 10 μL PBS with or without co-administration of tryptophan metabolites or vehicle. PBS was given in the right ear as control. Twenty-four hours later, the mice were injected intravenously with 0.5 mg of OVA in 200 μL 0.5% Evans Blue dye (Sigma-Aldrich) in PBS. 30 min later, the mice were euthanized by terminal anesthesia. Ear section was excised and dye was extracted with formamide at 55°C for 24 h. Absorbance was measured at 620 nm. PCA response was quantified by amount of dye leaked at IgE-injected sites. Data are expressed as Evans Blue dye in ng/mg tissue.

Intracellular calcium measurement. IgE-sensitized BMMCs were loaded with 8 $\mu\text{g/mL}$ Fluo-3-AM (Invitrogen) and 15 $\mu\text{g/mL}$ Fura red-AM (Invitrogen) for 30 min at 37°C . Cells were then washed twice and resuspended in Tyrode's buffer. OVA (10 $\mu\text{g/mL}$) was added for cell stimulation, and the resulting changes in intracellular calcium were monitored by FACSCalibur (BD Biosciences) and expressed as the fluorescent intensity of Fluo-3/Fura Red ratios over time.

Immunoblotting. Mast cells were lysed in RIPA buffer (Thermo Scientific) supplemented with protease inhibitor cocktails (BD Biosciences) and PhosSTOP (Roche). Cell lysates were directly analyzed followed by immunoblotting. For the detection of phosphorylated and total proteins, samples were loaded on the NuPAGE[®] Bis-Tris Mini Gels (Invitrogen) and transferred to PVN membrane. After the transfer to the membrane, the membrane was cut and each group of samples was detected for phosphorylated and total proteins. Proteins reactive with primary Abs were visualized with an HRP-conjugated secondary Ab (Santa Cruz Biotechnology) and ECL reagents (Western Lightning ECL Pro, PerkinElmer). Anti-PLC γ 1, anti-Akt, anti-p38 MAPK, anti-SAPK/JNK, anti-Erk1/2, anti-phospho-PLC γ 1, anti-phospho-Akt (Ser473), anti-phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-phospho-Erk1/2 (Thr202/Tyr204) Abs were from Cell Signaling Technology. Anti-actin Ab was from Serologicals Corporation.

Generation of human peripheral blood-derived cultured mast cells (HCMCs). Human cultured mast cells were generated *in vitro* from the peripheral blood mononuclear cells of 4

volunteers with informed consent and the protocol approved by the Institutional Review Board of National Health Research Institutes and Kaohsiung Medical University Hospital, Taiwan, following a negative selection protocol as described previously,¹ with the use of the lineage cell depletion kit containing a mixture of magnetic microbeads-conjugated Abs against human CD4, CD8, CD11b, CD14, and CD19 (Miltenyi Biotec). The remaining cell suspension was then incubated in Iscove's methylcellulose medium (StemCell Technologies) containing human SCF (200 ng/mL; R&D), IL-6 (50 ng/mL; R&D), and IL-3 (5 ng/mL; R&D). After 14 days in culture, fresh methylcellulose medium containing the same concentrations of SCF, IL-6, and IL-3 was layered over the methylcellulose cultures. At 6 weeks, the cells were then cultured in the presence of 100 ng/mL SCF, 50 ng/mL IL-6, 0.1% BSA, insulin-transferrin-selenium, and 55 μ M 2-ME, followed by flow cytometric analyses of the cells to verify the purity of the mast cell population by the use of anti-human CD117 (eBioscience) and Fc ϵ RI Abs (eBioscience).

Degranulation and IL-13 production in HCMCs. For analysis of IgE-mediated degranulation and IL-13 production in HCMCs, HCMCs (5×10^5 cells/mL) were sensitized with 1 μ g/mL purified human myeloma IgE (Millipore) in the presence of 50 μ M tryptophan metabolites, or vehicle control for 16 h, followed by washings with Tyrode's buffer and challenge with 3 μ g/mL anti-human IgE (Millipore) for 30 minutes. Subsequent analysis of degranulation was measured by the levels of β -hexosaminidase release as in the case of BMMCs. To determine the levels of IL-13,² HCMCs were first cultured with SCF and IL-6 in the presence of 10 ng/mL IL-4 for 5 days. After washing, cells were sensitized with 1 μ g/mL myeloma IgE in the presence of vehicle, 50 μ M KYN, or a combination of KYN (50 μ M) and CH223191 (10 μ M), a AhR antagonist, for 16 h, and followed by challenge with 3 μ g/mL anti-human IgE for 24 h. Human IL-13 in supernatant was measured by human IL-13 ultrasensitive ELISA kit (Invitrogen) according to the manufacturer's instructions.

Statistical analysis. Data were expressed as the means $\pm$ SEM. IBM SPSS Statistics ver. 19 was used for statistical analysis. P values of less than 0.05 were considered significant. The number of independent experiments is indicated in the figure legends. Where error bars are not presented, the data shown are representative of three independent experiments.

Supplementary References

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Supplementary Figure Legends

Supplementary Figure 1. Kynurenic acid (KA) and quinolinic acid (QA) do not potentiate IgE-mediated mast cell activation *in vitro*. (A, B) IgE-mediated degranulation. BMMCs from C57BL/6 mice were sensitized with anti-OVA IgE (E-C1) $\pm$ 0.1-1000 μ M KA (A), QA (B) or vehicle as control for 16 h, then stimulated with 0.5 μ g/mL OVA for 30 min. Degranulation was monitored by the release of β -hexosaminidase. Data are representative of two independent experiments. (C, D) IgE-mediated IL-13 production. BMMCs were sensitized with E-C1 $\pm$ 10-1000 μ M KA (C), QA (D) or vehicle for 16h, then washed, and stimulated with 3 μ g/mL OVA for 6 h. Data are representative of three independent experiments.

Supplementary Figure 2. Combination treatments of kynurenine and kynurenic acid (KYN+KA), quinolinic acid (KYN+QA), or a combination of all three metabolites (KYN+KA+QA) did not affect the enhancing effects by kynurenine on IgE-mediated mast cell activation. (A) IgE-mediated degranulation. BMMCs were sensitized with anti-OVA IgE (E-C1) in the presence of 50 μ M KYN and 50 μ M KA, 50 μ M KYN and 50 μ M QA, or 50 μ M KYN, 50 μ M KA and 50 μ M QA for 16 h, then washed, and stimulated with 3 μ g/mL OVA for 30 min. KA and QA were dissolved in PBS. Degranulation was monitored by the release of β -hexosaminidase. *** p <0.001 vs. KYN-treatment. n.s.; not significant. Data are representative of three independent experiments. (B) IgE-mediated LTC₄ release. BMMCs were sensitized with E-C1 in the presence of KYN, and KA or QA as above for 16h, then washed, and stimulated with 3 μ g/mL OVA for 30 min. * p <0.05, ** p <0.01 vs. KYN-treatment. n.s.; not significant. Data are representative of three independent experiments. (C) IgE-mediated IL-13 production. BMMCs were sensitized with E-C1 in the presence of KYN, and KA or QA as above for 16 h, then washed, and stimulated with 3 μ g/mL OVA for 6 h. *** p <0.001 vs. KYN-treatment. n.s.; not significant. Data are representative of three independent experiments.

Supplementary Figure 3. Expression level of FcεRI on BMMCs after treatment of kynurenine. BMMCs were treated with E-C1 and 50 μM KYN for 16 h and then measured the expression of FcεRI by flow cytometry with Abs specific for FcεRI (MAR-1; eBiosciences).

Supplementary Figure 4. Kynurenine level in culture medium. Amount of kynurenine in the culture media in the presence or absence of tryptophan was measured with a Kynurenine ELISA kit (BlueGene) according to the manufacturer's instructions. Detection limit of the kit is around 50 nM. Data are representative of two independent experiments.