

BRIEF COMMUNICATION

Skin thymic stromal lymphopoietin promotes airway sensitization to inhalant house dust mites leading to allergic asthma in mice

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Abstract

Asthma is often preceded by atopic dermatitis (AD), a phenomenon known as 'atopic march'. It has been suggested that sensitization to common inhalant allergens, which is developed in a majority of patients with AD and often during the course of AD, may play a critical role in triggering the atopic march. Yet, what signal(s) delivered by AD skin could promote sensitization to inhalant allergens remains elusive. Here, by employing an experimental mouse asthma protocol, which is induced by airway sensitization and challenge to inhalant house dust mite (HDM), we demonstrate that the overproduction of cytokine thymic stromal lymphopoietin (TSLP) by AD skin promotes airway sensitization to HDM, thereby triggering subsequently an allergic asthma. Together, this study provides, for the first time, the experimental proof that TSLP represents an AD skin-delivered signal to promote sensitization to inhalant aeroallergen, which may account for one mechanism underlying the 'atopic march'.

Asthma is often preceded by atopic dermatitis (AD), a phenomenon known as 'atopic march' (1, 2). It has been recognized that sensitization to common inhalant allergens is developed in a majority of patients with AD, often during the course of AD (1, 3). Moreover, an early onset and the severity of AD are associated with the degree of sensitization to aeroallergens, and with the risk of developing asthma (2). Although these studies have suggested that AD skin may deliver signal(s) that could promote sensitization to inhalant allergens (3), thus triggering the onset of 'atopic march', the involved mechanism remains to be clarified.

Thymic stromal lymphopoietin (TSLP) produced by skin keratinocytes has emerged as a key player in AD pathogenesis (4, 5). Increased expression of TSLP has been revealed in epidermal keratinocytes from patients with AD (6), and elevated serum TSLP level was reported in children with AD (7). TSLP overproduction has been also reported in the skin of patients with Netherton disease, which is highly associated with AD and atopy (8). Our previous studies using mouse

models have shown that TSLP overexpression in keratinocytes not only triggers a spontaneous AD-like dermatitis, but also aggravates experimental allergic asthma induced upon intraperitoneal (i.p.) sensitization to ovalbumin (OVA) and airway OVA challenge (9). This was supported by the study of Demehri, et al. (10). Importantly, employing a TSLP^{over} protocol in which TSLP expression in keratinocytes is induced by skin topical application of MC903 (a vitamin D3 analog) (11, 12), we showed that overproduction of keratinocytic TSLP during the i.p. sensitization phase could aggravate OVA asthma (9). This study suggested that TSLP produced by AD skin enters the circulation and acts as a skin-derived signal to promote allergen sensitization, thereby contributing to the 'atopic march' (9). However, it remained unclear whether these findings have clinical relevance. Indeed, the i.p. sensitization of OVA complexed with exogenous Th2 adjuvant (alum) is far from a 'real-life' situation, and moreover, OVA is not a natural inhalant allergen, and it does not prime airway sensitization the same way as house dust mites

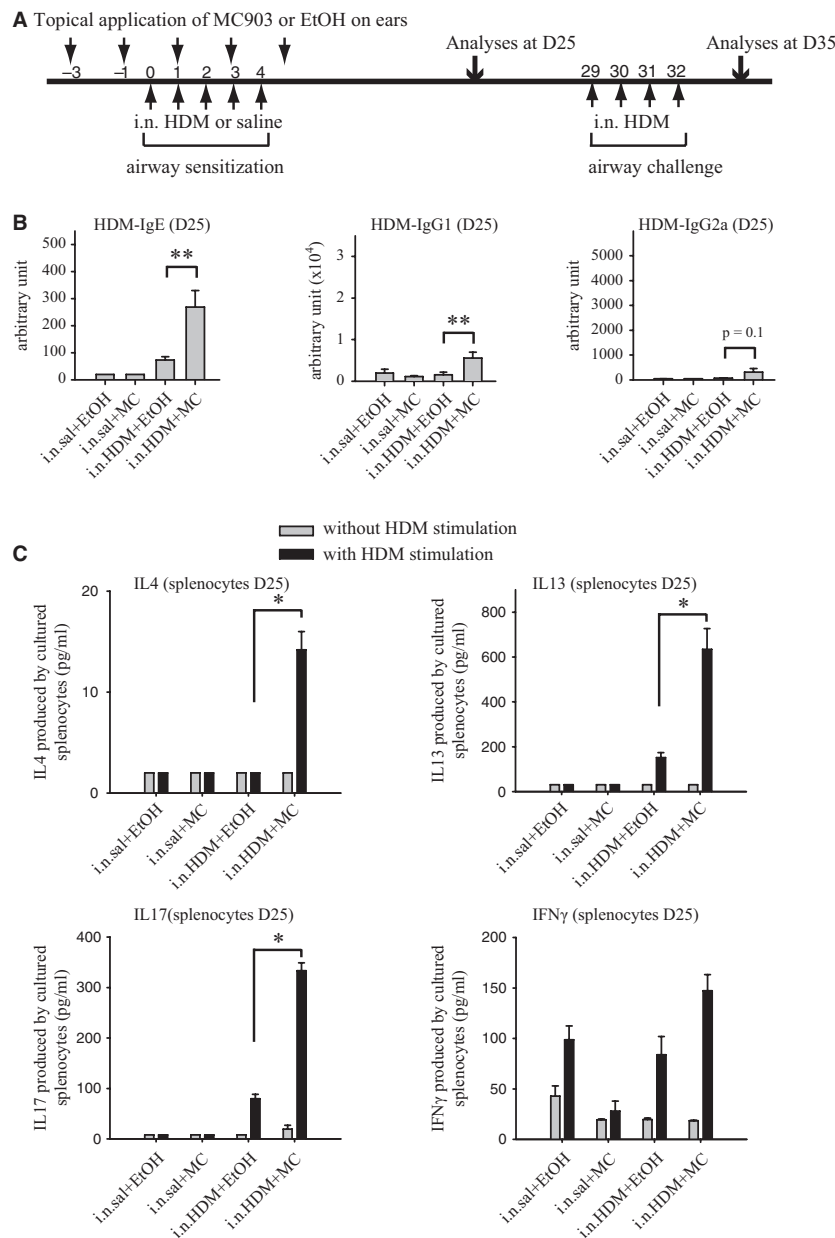


Figure 1 Skin MC903 topical application enhances airway sensitization to house dust mites (HDM). (A) Experimental protocol. Wild-type Balb/c mice were intranasally (i.n.) sensitized with HDM (0.5 μ g) or saline (sal; vehicle control) (once per day from D0 to D4), and i.n. challenged with HDM (0.05 μ g) 3 weeks later (once per day from D29 to D32). To induce thymic stromal lymphopoietin (TSLP) overproduction in skin keratinocytes during the HDM

sensitization phase, MC903 (MC, 1 nmol) was topically applied on mouse ears (once every other day from D-3 to D5). Ethanol (EtOH) application was used as vehicle control. (B) Serum HDM-specific IgE, IgG1, and IgG2a at D25 (after i.n. sensitization, but before i.n. challenge). (C) Cytokine production by splenocytes from mice at D25, without or upon HDM stimulation. * $P < 0.05$, ** $P < 0.01$ ($n = 6$ mice per group). Values are mean $\pm$ SEM.

(HDM), pollens and other aeroallergens do in human asthma (13, 14).

We thus set off to determine whether TSLP overproduced in AD skin could impact sensitization to inhaled aeroallergens and thereby triggers the development of allergic asthma. The experimental protocol is outlined in Fig. 1A, in which an initial sensitization via intranasal (i.n.) exposure of the clinically

relevant aeroallergen HDM, followed by i.n. challenge of HDM 3 weeks after. This protocol allowed us to temporally dissect the phases of airway sensitization and challenge. To effectively reveal the effect of skin-derived TSLP on airway sensitization, we used here a 'sub-asthmatic' mild HDM dose (0.5 μ g for sensitization and 0.05 μ g HDM for challenge), which induced in wild-type Bab/c mice a weak sensitization to

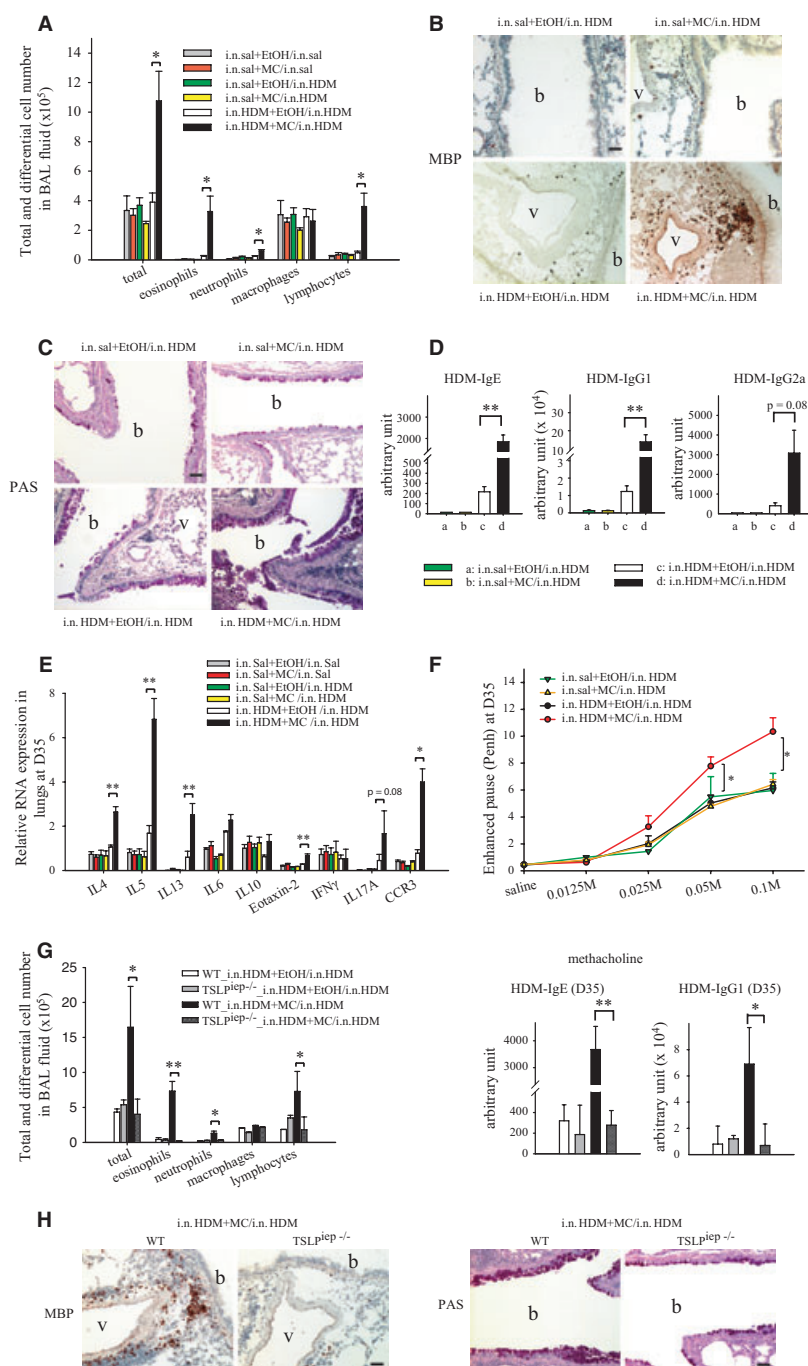


Figure 2 Overproduced keratinocytic thymic stromal lymphopoietin (TSLP) during HDM airway sensitization phase triggers an aggravated allergic airway inflammation upon HDM challenge. (A) Cell counts in the bronchoalveolar lavage (BAL) fluid at D35. $*P < 0.05$ ($n = 6$ mice per group). Values are mean $\pm$ SEM. (B) Immunohistochemistry staining with antibody against MBP (major basic protein; specific for eosinophils), and (C) Periodic acid-Schiff (PAS) staining (for mucus-secreting goblet cells) of lung sections at D35. b, bronchiole; v, vessel. Scale bars, 50 μm . (D) Serum HDM-specific IgE, IgG1, and IgG2a at D35. (E) Quantitative RT-PCR analyses of cytokines and chemokines in lungs at D35. (F)

Airway hyperresponsiveness (AHR) evaluated by plethysmography after exposure to increased dose of methacholine at D35. $*P < 0.05$. ($n \geq 6$ mice per group). (G) Comparison of cell counts in the BAL fluid (left panel), and of serum HDM-specific IgE and IgG1 levels (right panel) of wild-type control (WT) and TSLP^{iepi} mice at D35, upon i.n.HDM + EtOH/i.n.HDM or i.n.HDM + MC/i.n.HDM treatment. $*P < 0.05$ ($n = 6$ mice per group). Values are mean $\pm$ SEM. (H) Comparison with MBP IHC staining (left panel) and PAS staining (right panel) of lung sections of i.n.HDM + MC/i.n.HDM-treated WT and TSLP^{iepi} mice at D35. Scale bars, 50 μm .

HDM (see Fig. 1B), and generated a very mild allergic asthma upon i.n. HDM challenge (see Fig. 2A–F). During the phase of airway sensitization, TSLP production in skin keratinocytes was induced by MC903 topical application on mouse ears (9) (Fig. 1A). Note that MC903 treatment induced TSLP production in the skin, with simultaneously increased serum TSLP levels (9), whereas ending the MC903 treatment normalized skin and serum TSLP levels within 3 days [ref (9) and data not shown].

We first examined the effect of skin MC903 treatment on the sensitization to HDM. At D25 (a time-preceding airway challenge), the i.n.HDM + MC (i.n. HDM-sensitized and skin MC903 treated) mice exhibited much higher HDM-specific IgE and IgG1 in sera, compared with i.n. HDM + EtOH (i.n.HDM-sensitized and skin EtOH treated) mice (Fig. 1B). *In vitro* HDM stimulation of splenocytes from these mice induced much higher production of Th2 cytokines (IL4 and IL13), as well as Th17 cytokine (IL17), but not Th1 cytokine (IFN γ) (Fig. 1C). Therefore, skin topical MC903 application enhances Th2 and Th17 sensitization to inhaled HDM.

We then analyzed pulmonary inflammation upon HDM challenge. Whereas i.n.HDM + EtOH/i.n.HDM mice exhibited only a mild airway inflammation, the i.n. HDM + MC/i.n.HDM mice developed a robust asthmatic phenotype at D35. Indeed, bronchoalveolar lavage (BAL) cell counting showed a significant increase of eosinophils, neutrophils and lymphocytes in the i.n.HDM + MC/i.n.HDM mice (Fig. 2A). These mice exhibited much more severe pulmonary infiltration of eosinophils (Fig. 2B) and CD4⁺ T cells (but not CD8⁺ T cells, data not shown), as well as stronger mucus production (Fig. 2C). They produced much higher HDM-specific IgE and IgG1 in sera (Fig. 2D). RT-PCR analyses of lungs of these mice showed that the expression of Th2 cytokines (IL4, IL5, IL13), eosinophil-attractant chemokine (eotaxin-2), and receptor (CCR3) was all increased. The expression of Th17 cytokine (IL17A) showed a tendency to increase (although the statistic analyses did not reach significance), whereas Th1 cytokine (IFN γ) expression remained unchanged (Fig. 2E). Moreover, the i.n.HDM + MC/i.n. HDM mice also exhibited an enhanced airway hyperresponsiveness (Fig. 2F). Notably, in the absence of HDM sensitization, MC903 did not induce any lung inflammation upon i.n. HDM challenge (see i.n.sal + MC/i.n.HDM in Fig. 2A–F). It suggests that the promotion of allergic asthma by skin MC903 requires the copresence of allergen sensitizing. Together, these results indicate that MC903 application in the skin during the HDM airway sensitization phase triggers an aggravation of Th2- and Th17- (to a lesser extent) type allergic asthma upon HDM airway challenge.

To examine whether the MC903-induced aggravation of HDM asthma is mediated by keratinocytic TSLP, we employed TSLP^{iep-/-} mutant mice in which TSLP gene is selectively and inducibly ablated in epidermal keratinocytes (11) (see Fig. S1). As expected, MC903-enhanced accumulation of eosinophils, neutrophils, and lymphocytes in BAL fluid, increase of HDM-specific IgE and IgG1, pulmonary infiltrate of eosinophils, as well as mucus production in

HDM-treated wild-type (WT) mice, were all abolished in HDM-treated TSLP^{iep-/-} mutant mice (Fig. 2G–H). Moreover, MC903-enhanced AHR in HDM-treated WT mice was also diminished upon TSLP ablation in keratinocytes (see Fig. S2). These results thus demonstrate that TSLP overproduced by skin keratinocytes promotes airway sensitization to HDM and thereby triggers subsequently an HDM allergic asthma.

Collectively, we provide here for the first time the experimental proof that TSLP represents an AD skin-delivered signal to promote airway sensitization to inhaled aeroallergen like HDM, which may account for one mechanism underlying the 'atopic march'. We find that skin TSLP enhances not only HDM-primed Th2 but also Th17 immune responses (15), while barely affecting Th1 response. Of interest, we observed additionally that TSLP could promote sensitization to very low 'non-asthmatic' dose of HDM (e.g. 0.1 μ g) (data not shown). Given that HDM is the most clinically relevant aeroallergen for allergic asthma and that its levels in the environment have been correlated with prevalence of asthma (16), our finding is of great clinical implication. It suggests that when exposed to common environment, AD individuals bearing increased skin TSLP expression may have a higher risk for developing sensitization to inhalant common allergens through airways, which contributes to the onset of the 'atopic march'. It will be necessary to examine whether serum levels of TSLP in patients with AD, particularly in the infants with early-onset AD (in which sensitization often develops at early stage) (3), correlate with sensitization to environmental allergens in these patients. Our study also suggests that treatment or prevention strategy of asthma only targeting on airway may be insufficient, and blocking TSLP production in skin could be therapeutically useful in preventing or limiting airway sensitization, which is commonly developed in patients with AD, and halting the progress of the 'atopic march'.

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Conflict of interest

The authors have declared that no conflict of interest exists.

Authors contributions

Hua Jiang and Mei Li designed experiments, analyzed the data, and wrote the manuscript. Hua Jiang and Pierre Hener performed mouse experiments, BAL analyses, histology and immunohistochemistry, Elisa, RT-PCR, and airway reactivity analyses. Jiagui Li performed Elisa analyses of HDM-specific Igs and cytokines.

Supporting Information

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

Data S1. Methods.

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Supporting Information

Supplementary Figure S1. Comparison of serum TSLP levels in wildtype controls (WT) and TSLP^{iep-/-} mutant mice at D7 (see Fig. 1A for experimental protocol), showing the abolished TSLP production in sera of the TSLP^{iep-/-} mice upon topical application of MC903 (MC) on ears. TSLP^{iep-/-}, mice with ablation of TSLP selectively and inducibly in epidermal keratinocytes, which were generated upon tamoxifen (Tam) treatment of K14-Cre-ER^{T2(tg/0)}/TSLP^{L2/L2} mice. Tam-treated age- and sex-matched littermates (K14-Cre-ER^{T2(0/0)}/TSLP^{L2/L2}) were used as WT controls.

Supplementary Figure S2. Airway hyperresponsiveness (AHR) evaluated by plethysmography after exposure to increased dose of methacholine at D35. *p<0.05. (n=6 mice per group). TSLP^{iep-/-}, mice with ablation of TSLP selectively and inducibly in epidermal keratinocytes. WT, wildtype controls.

Supplementary Methods

Mice

Ten-to-twelve-week-old wild-type female BALB/c mice were purchased from Charles River Laboratories. Six-to-eight-week-old K14-Cre-ER^{T2(tg/0)}/TSLP^{L2/L2} female mice (in a Balb/c background) were injected intraperitoneally with tamoxifen (Tam, 0.1 mg in 100 µl sun flower oil, for five consecutive days), to generate TSLP^{iep-/-} mice (1, 2). Tam-injected age- and sex-matched littermates (K14-Cre-ER^{T2(0/0)}/TSLP^{L2/L2}) were used as wild-type controls. TSLP^{iep-/-} and wildtype control

mice were subjected to experiments 4 weeks after the first Tam injection. Breeding and maintenance of mice were performed under institutional guidelines, and all of the experimental protocols were approved by the Animal Care and Use Committee of the Institut de Génétique et de Biologie Moléculaire et Cellulaire.

HDM i.n. sensitization and i.n. challenge

House dust mite (HDM) (*Dermatophagoides pteronyssinus*) extracts were obtained from Greer Laboratories (Lenoir, NC) as a lyophilized preparation of milled mites. Mice were sensitized by intranasal (i.n.) instillation with vehicle (0.9% sterile saline) or HDM extracts on days (D) 0, 1, 2, 3 and 4 (0.5 µg of total protein in 25 µl). On D29, 30, 31 and 32, mice were i.n. challenged with HDM extract (0.05 µg of total protein in 25 µl).

MC903 topical application

MC903 (calcipotriol; Leo Pharma) was dissolved in ethanol (EtOH) and topically applied on mouse ears (1 nmol in 25 µl per ear) at D -3, -1, 1, 3, and 5 (3). As vehicle control, EtOH was topically applied on mouse skin.

Histopathology

Lung tissues were fixed overnight at 4 °C in 4% paraformaldehyde (PFA), and embedded in paraffin. Sections (3µm) were stained with hematoxylin/eosin (H&E) or Periodic acid-Schiff (PAS; for goblet cells).

Immunohistochemical (IHC) staining

For IHC staining of MBP (major basic protein; specific for eosinophils), 3 µm

paraffin sections were treated with 0.6% H₂O₂ to block the endogenous peroxidase activity, followed by digestion with Pepsin solution (Invitrogen) in order to retrieve antigen. Slides were then blocked with rabbit serum (Vector Laboratories), and incubated with rat-anti-mouse MBP (provided by Dr James J Lee, Mayo Clinic, Rochester, USA) antibody. After incubation with biotinylated rabbit anti-rat IgG, followed by AB complex (Vector Laboratories), staining was finally visualized with AEC+ high sensitivity substrate chromogen solution (Dako) and counterstained with hematoxylin.

Serum HDM-specific immunoglobulin (Ig) determination

Microtiter plates (R&D Systems) were coated with HDM (10 µg/ml in 0.1M sodium bicarbonate buffer). After blocking with 1% BSA (Sigma), the coated plates were then incubated with serum samples, followed by incubation with a biotinylated rat anti-mouse IgE (clone R35–118, BD Biosciences PharMingen), rat anti-mouse IgG1 (clone A85-1, BD Biosciences PharMingen), or rat anti-mouse IgG2a (clone R19-15, BD Biosciences PharMingen). ExtrAvidin peroxidase (Sigma) and TMB (tetramethylbenzidine) Substrate Reagent Set (BD Biosciences PharMingen) were used for detection. Serum levels of HDM-specific Igs were related to a pooled serum from HDM-sensitized and challenged BALB/c mice (internal standard) and expressed as arbitrary units.

Cell culture and in vitro cytokine production

Splenocytes (4.5×10^6 /well) were cultured in the presence of 50 µg/ml HDM for 5 days. Culture supernatants were then harvested, and cytokine production of IL4, IL5, IL13, IL17 and IFN γ was measured using DuoSet ELISA Development system (R&D

systems) according manufacturer's instructions.

Quantitative RT-PCR

RNA was reverse-transcribed using random oligonucleotide hexamers and amplified by quantitative PCR with a Ligthcycler 480 (Roche Diagnostics) and the QuantiTect SYBR Green kit (Qiagen), according to the manufacturer's instructions. Hypoxanthine phosphoribosyltransferase (HPRT) was used as an internal control. Sequences of PCR primers: HPRT, TSLP, IL4, IL5, IL13, IFN γ , IL10, IL6, Eotaxin-2 and CCR3 were as previously reported (4); IL17A (239bp) (5'-CCAGGGAGAGCTTCATCTGT-3'; 5'-ACGTGGAACGGTTGAGGTAG-3').

Airway Reactivity.

Airway reactivity was measured by noninvasive whole-body plethysmography (Emka Technologies, France) in response to inhaled methacholine (Sigma), using enhanced pause (Penh) as an index of airway responsiveness.

Statistic analysis

Data are represented as mean $\pm$ S.E. and were analyzed using SigmaStat (Systat Software Inc. Point Richmond, CA, USA) by the Student t test or the Mann-Whitney rank sum nonparametric test depending on results from the Kolmogorov-Smirnov test (with Lilliefors' correction) for normality and Levene Median test for equal variance. Two-way repeated measures ANOVA was used to compare airway AHR between different groups responding to various doses of methacholine.

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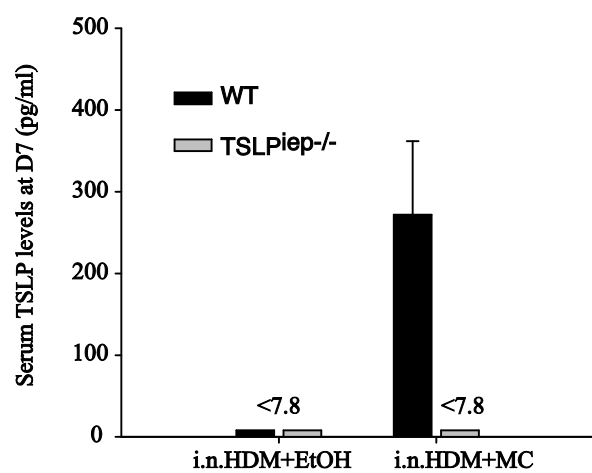


FIG. S1

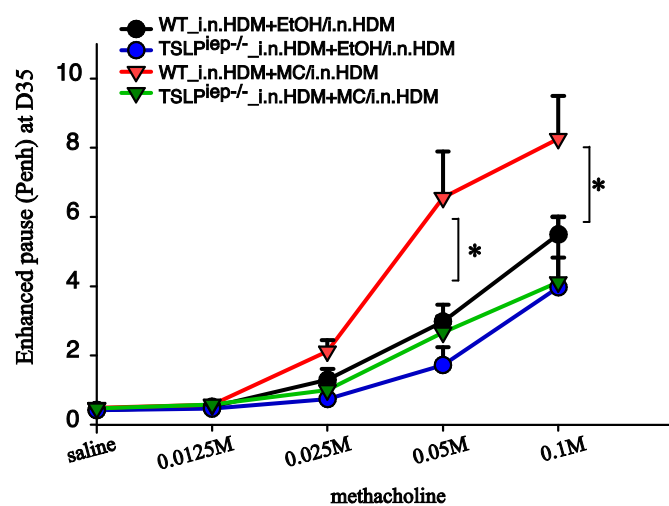


FIG. S2