Mast cells instruct keratinocytes to produce thymic stromal lymphopoietin: Relevance of the tryptase/protease-activated receptor 2 axis

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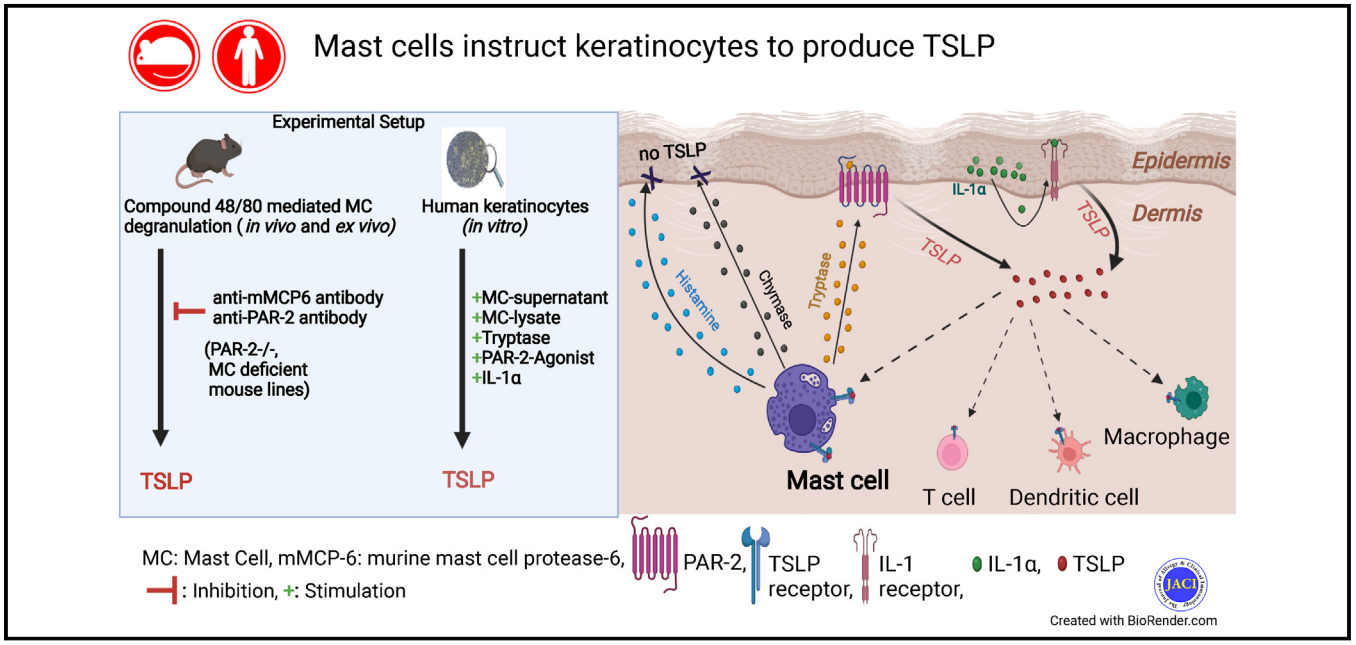
GRAPHICAL ABSTRACT



Background: Thymic stromal lymphopoietin (TSLP) promotes T_H2 inflammation and is deeply intertwined with inflammatory dermatoses like atopic dermatitis. The mechanisms regulating TSLP are poorly defined.

Objective: We investigated whether and by what mechanisms mast cells (MCs) foster TSLP responses in the cutaneous environment.

Methods: *Ex vivo* and *in vivo* skin MC degranulation was induced by compound 48/80 in wild-type protease-activated receptor 2 (PAR-2)- and MC-deficient mice in the presence or absence of neutralizing antibodies, antagonists, or exogenous mouse MC protease 6 (mMCP6). Primary human keratinocytes and murine skin explants were stimulated with lysates/supernatants of human skin MCs, purified tryptase, or MC lysate diminished of tryptase. Chymase and histamine were also used. TSLP was quantified by ELISA, real-time quantitative PCR, and immunofluorescence staining.

Results: Mas-related G protein–coupled receptor X2 (Mrgprb2) activation elicited TSLP in intact skin, mainly in the epidermis. Responses were strictly MC dependent and relied on PAR-2. Complementarily, TSLP was elicited by tryptase in murine skin explants. Exogenous mMCP6 could fully restore responsiveness in MC-deficient murine skin explants. Conversely, PAR-2 knockout mice were unresponsive to mMCP6 while displaying increased responsiveness to other inflammatory pathways, such as IL-1α. Indeed, IL-1α acted in concert with tryptase. In primary human keratinocytes,

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MC-elicited TSLP generation was likewise abolished by tryptase inhibition or elimination. Chymase and histamine did not affect TSLP production, but histamine triggered IL-6, IL-8, and stem cell factor.
Conclusion: MCs communicate with keratinocytes more broadly than hitherto suspected. The tryptase/PAR-2 axis is a crucial component of this cross talk, underlying MC-dependent stimulation of TSLP in neighboring keratinocytes. Interference specifically with MC tryptase may offer a treatment option for disorders initiated or perpetuated by aberrant TSLP, such as atopic dermatitis. (J Allergy Clin Immunol 2022;■■■:■■■-■■■.)

Key words: *Atopic dermatitis, interleukin 1, keratinocytes, mast cells, PAR-2, TSLP, tryptase*

Though mast cells (MCs) are commonly increased in inflammatory dermatoses, including atopic dermatitis (AD) and psoriasis, and are purportedly linked to the underlying pathology,[1-4] the mechanisms by which MCs initiate or perpetuate cutaneous disorders are not well understood. By their localization close to the basement membrane in the upper dermis and wide range of soluble mediators, MCs are excellently positioned to engage in epidermal–dermal cross talk, although knowledge about MC–epidermal communication is still limited. In fact, MC-initiated processes in keratinocytes (KCs) are mostly limited to biogenic amines (histamine, serotonin) reported to increase KC proliferation or cytokine production.[5-7]

Thymic stromal lymphopoietin (TSLP) is associated with multiple skin disorders and is mainly produced by epithelial and some stromal cells.[8-10] As a result of its T_H2-skewing potential, the cytokine has been most intimately connected with AD and asthma.[8,9] As a consequence, TSLP-targeted therapies have recently entered clinical trials.[11,12] In addition, TSLP acts as a sensitive indicator of even slight deviations from homeostasis, including those provoked by mild physical or chemical irritation of the skin, a setting to which PAR-2 activation contributes.[13,14] PAR-2 is widely expressed in skin and is prominent in KCs,[15-17] where it regulates proliferation and differentiation,[18] inflammation, and pruritus.[17,19] In fact, PAR-2 ligation by tryptase, a preformed serine protease abundantly and specifically expressed by MCs that is released by MC degranulation,[20,21] can stimulate Ca^{2+} responses in KCs *in vitro*.[22,23] PAR-2 activation *in vivo* by epidermal kallikreins is associated with TSLP induction in atopic-like conditions.[24,25]

MCs have been reported to produce TSLP upon stimulation[26,27] but also to facilitate TSLP production by epithelial cells[28] and in the context of helminth infection.[29] However, the mediators and mechanisms by which MCs dictate TSLP generation in neighboring cells are poorly understood, and especially little is known for the skin. The current study revealed that cutaneous MCs elicit TSLP responses in intact skin and in primary KCs. By combining *in vitro, ex vivo,* and *in vivo* approaches, we found that TSLP generation critically depends on the MC tryptase/PAR-2 axis, but not on histamine or chymase. An intact tryptase/PAR-2 system also drives TSLP stimulation in cells of human skin origin. We conclude that tryptase is an endogenous key player regulating TSLP in the cutaneous environment, suggesting that it may serve as a target in inflammatory dermatoses, including AD.

Abbreviations used

AD:	Atopic dermatitis
c48/80:	Compound 48/80
ELISA:	Enzyme-linked immunosorbent assay
hKC:	Human primary keratinocyte
HPRT:	Hypoxanthine–guanine phosphoribosyltransferase
IF:	Immunofluorescence
IL-1RA:	IL-1 receptor agonist
IP:	Immunoprecipitation
KC:	Keratinocyte
KO:	Knockout
MC:	Mast cell
mMCP6:	Mouse MC protease 6
Mrgprb2/MRGPRX2:	Mas-related G protein–coupled receptor X2
mRNA:	Messenger RNA
mSE:	Murine skin explant
PAR-2:	Protease-activated receptor 2
qPCR:	Real-time quantitative PCR
SCF:	Stem cell factor
SLIGRL-NH_2:	PAR-2 activating peptide
TSLP:	Thymic stromal lymphopoietin
WT:	Wild type

METHODS

Mice

We purchased 8- to 10-week-old female C57BL/6 wild-type (WT) and PAR-2 knockout (KO) mice from Janvier (Saint-Berthevin, France) and The Jackson Laboratory (Bar Harbor, Me), respectively. MC-deficient mice were generated and bred in the Charité animal facility. The C57BI/6J-Mcpt5-Cre and the B6;129S7-Dicer^{1tm1Smr}/JxB6 mouse strains were as described.[30] The C57BI/6J-Mcpt5-Cre strain was bred as WT × heterozygous and crossed to homozygous animals of the B6;129S7-Dicer^{1tm1Smr}/JxB6 strain, resulting in the second generation in MC-deficient mice and Cre-negative littermates homozygous for the *Dicer* gene. The latter age-matched animals were used as controls. All animal experiments were performed as approved by the local state office of health and social affairs (LaGeSo, G0262/15) and performed in agreement with their protocols.

Skin explant treatment

Murine skin explants (mSEs) were treated as described in the Methods section in this article's Online Repository (available at www.jacionline.org).

In vivo MC degranulation

Belly skin was wet shaved 48 hours before c48/80 administration. Each mouse was intradermally injected with 20 μg of anti-mMCP6 (or goat-IgG). Desloratadine and JNJ maleate were applied by oral gavage (phosphate-buffered saline and dimethyl sulfoxide served as vehicle controls, respectively). After 14 hours, mice were intradermally injected with c48/80 (a total of 100 μg per mouse), and 8 hours later, mice were anesthetized, and after blood collection were then humanely killed. Skin was frozen for further analysis.

Immunofluorescence staining

Immunofluorescence (IF) was performed as described in the Methods section this article's Online Repository.

Skin lysis

Skin was homogenized by prechilled Precellys homogenizer (Peqlab, Erlangen, Germany) in lysis buffer (2 mmol/L EDTA, 1 mmol/L

dithiothreitol, 25 mmol/L Tris [pH 7.8], 1% Triton X-100, and 10% glycerol) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland). Protein quantification was performed by the bicinchoninic acid assay (Pierce Laboratories, Rockford, Ill). TSLP concentration in skin extracts was quantified by ELISA.

Keratinocyte isolation and treatments

Human primary keratinocytes (hKCs) were isolated from foreskins as described.[31] The skin was obtained from circumcisions, with informed written consent of the patients or their legal guardians and approval by the university ethics committee (EA01/194/14). The experiments were conducted according to the Declaration of Helsinki principles.

The KCs were cultured in DermaLife K serum-free KC medium supplemented with essential factors along with 60 μmol $CaCl_2$ (Lifeline Cell Technology, Walkersville, Md). Upon reaching 70-80% confluency, cells were detached by trypsin-ethylenediaminetetraacetic acid (PAA Laboratories, Cölbe, Germany) and then 2.5×10^3 seeded per well in a 48-well plate to grow for 96 hours, followed by stimulation with MC lysate/supernatant, either alone or in the presence of nafamostat for 24 hours. Additionally, KCs were stimulated with histamine (10 μmol) or recombinant human skin β tryptase (15 ng/mL) alone or in combination with chymase (250 ng/mL) or IL-1α (2, 5, and 10 ng/mL) and/or recombinant human IL-1 receptor agonist (IL-1RA) (100 ng/mL). Furthermore, KCs were stimulated with a PAR-2 agonist, PAR-2 activating peptide (SLIGRL-NH_2; 100 μm), alone or in combination with IL-1α and/or recombinant human IL-1RA. Culture supernatants were collected after 24 hours, and TSLP or IL-8 levels were determined by ELISA. For messenger RNA (mRNA) expression, hKCs were incubated with stimuli for 2.5 hours.

Skin MC isolation and treatment

MCs were isolated from human foreskin tissue as described.[32,33] Skin was obtained from circumcisions as in the case of KCs (EA1/204/10). Skin MCs were lysed *ex vivo* by freeze–thaw cycles. Supernatants were taken after 30 minutes in basal Iscove medium with 10% fetal calf serum (Biochrom, Berlin, Germany) and used for the stimulation of KCs.

Removal of tryptase by immunoprecipitation and immunoblotting

Tryptase was removed from MC lysates by 3 rounds of immunoprecipitation (IP), as detailed in the Methods section in the Online Repository.

Reagents

Reagents were purchased from commercial sources, as detailed in the Methods section in the Online Repository.

Statistical analysis

Data are expressed as means ± SEMs. For more than 2 groups, 1-way ANOVA was used, followed by the Bonferroni multiple comparisons test or Kruskal-Wallis test combined with the Dunn multiple comparison test, depending on data distribution. The *t* test (paired or unpaired) or Wilcoxon matched paired test (when data were not normally distributed) were used to compare 2 groups. Analyses were performed by GraphPad Prism 6 (GraphPad Software, La Jolla, Calif). $P < .05$ was considered statistically significant.

Other methods

Detailed descriptions of ELISA, RNA isolation, and real-time quantitative PCR (qPCR) are listed in the Methods section in the Online Repository.

RESULTS

TSLP is elicited by MC activation in murine skin

To investigate the role of MCs in the generation of TSLP, we treated mSEs from MC-deficient and Dicer control mice with c48/80 (Fig 1, *A*), which activates Mas-related G protein–coupled receptor X2 (Mrgprb2; MRGPRX2 in humans).[34-36] The c48/80 efficiently stimulated TSLP in explants from MC-sufficient but not MC-deficient mice (Fig 1, *B*), suggesting that MCs can orchestrate TSLP generation in the skin. To verify the findings *in vivo,* c48/80 was intradermally injected, the skin was lysed and analyzed for TSLP accumulation (Fig 1, *C*). Again, the MC-deficient mice produced less TSLP compared to Dicer control mice (Fig 1, *D*).

These findings could have resulted from TSLP production by MCs themselves or from an indirect route, by which MCs instruct neighboring cells to generate TSLP in a paracrine manner. To distinguish between the possibilities, TSLP-expressing cells were analyzed by IF.[13] TSLP staining was predominantly epidermal and considerably more prominent in c48/80-treated skin than in phosphate-buffered saline–treated control skin, suggesting that MC activation was followed by activation of KCs (Fig 1, *E*). Hematoxylin and eosin staining of skin sections can be found in Fig E1 in the Online Repository at www.jacionline.org.

A tryptase/PAR-2 axis drives MC-dependent TSLP production in the skin

We set out to delineate the pathways by which MCs elicit TSLP in KCs. Because responses were rapid, we assumed that preformed MC mediators rather than (newly generated) cytokines were the relevant entities. Using the anti-mMCP6 antibodies desloratadine and JNJ maleate to interfere with tryptase (mMCP6 in the mouse) histamine receptor types 1 and 4, respectively (Fig 2, *A*), we determined that tryptase was crucially implicated in TSLP production, whereas histamine receptor antagonists had no effect (Fig 2, *B*). Interference with mMCP6 also blunted TSLP upregulation at the mRNA level (Fig 2, *C*).

Because tryptase can activate PAR-2 on KCs,[37] we studied the significance of PAR-2 by a complementary approach. In fact, while c48/80 induced robust TSLP in WT mice, the response was substantially weaker in PAR-2–deficient equivalents, indicating that a sizable fraction of the response to c48/80 required PAR-2 (Fig 2, *D* and *E*). To verify this, we used a strategy that bypasses MCs by applying mMCP6 directly. Again, exogenous mMCP6 stimulated TSLP only in PAR-2–sufficient explants (Fig 2, *F*). The same was observed when mMCP6 was substituted by a synthetic PAR-2 agonist (see Fig E2, *A,* in the Online Repository available at www.jacionline.org).

Collectively, acute MC stimulation in intact skin elicits TSLP generation in KCs by activating the tryptase/PAR-2 axis.

MC secretory products and exogenous tryptase stimulate TSLP via PAR-2 activation *ex vivo*

To assess whether MC secretory products can induce TSLP, mSEs were treated with supernatants derived from bone marrow–derived cultured MCs (which contain mMCP6).[38] Because MCs secrete preformed mediators spontaneously (albeit at lower level

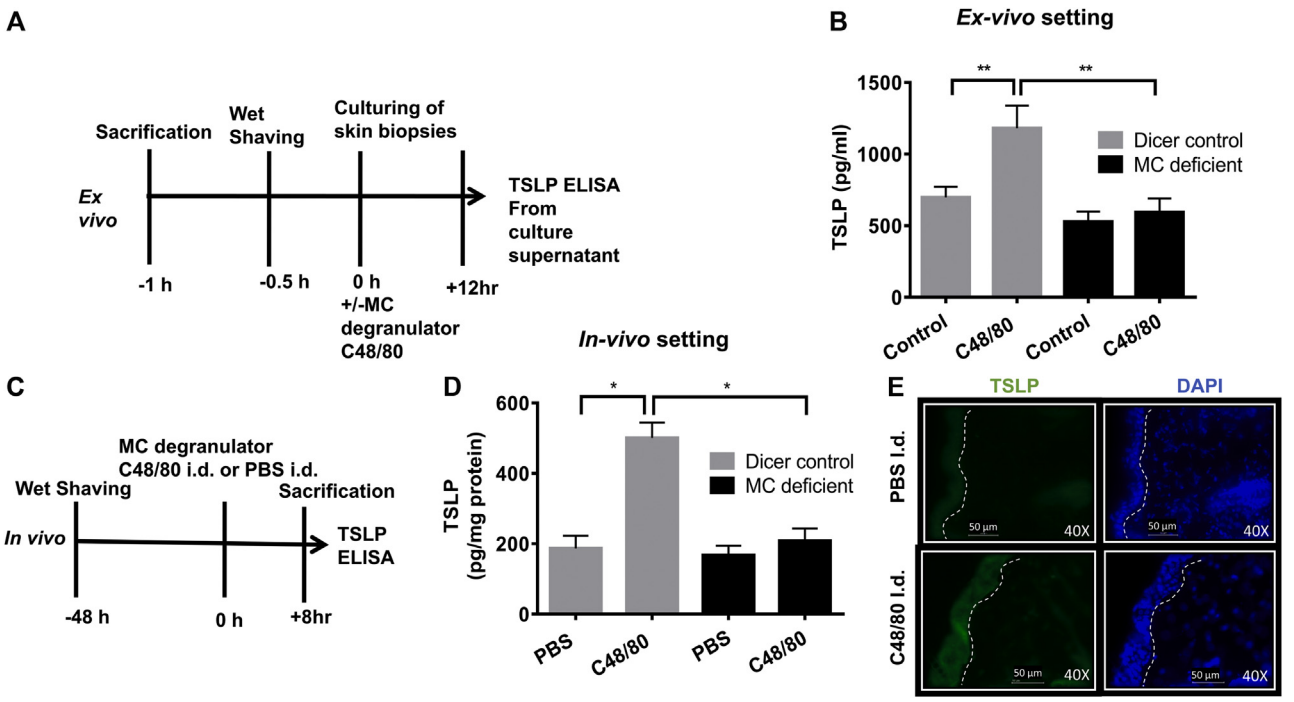

FIG 1. Stimulation of MCs in murine skin triggers TSLP. (**A, B**) *ex vivo* MC degranulation. (*A*) Scheme of model. (*B*) TSLP quantification in skin explants by ELISA. (**C-E**) *In vivo* MC degranulation. (*C*) Scheme of model. (*D*) TSLP quantification in skin lysates by ELISA. (*E*) Representative IF of skin sections (original magnification 40×). *Green* indicates TSLP; *blue,* DAPI. The *dotted line* demarcates the dermal–epidermal border. Means ± SEMs of 6-8 experiments/mice. **P* < .05, ***P* < .01. *DAPI,* 4′,6-Diamidino-2-phenylindole.

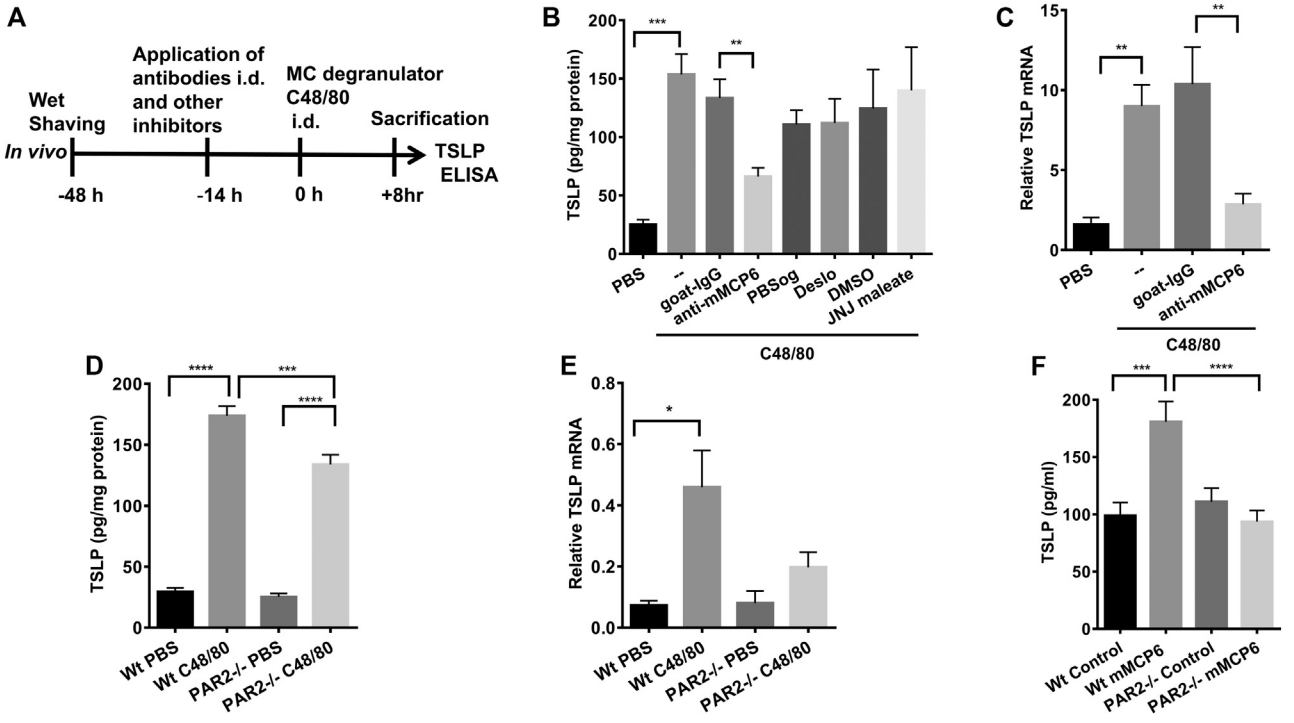

FIG 2. A tryptase/PAR-2 axis drives MC-dependent TSLP production in the skin. (**A**) Scheme of *in vivo* model. (**B** and **C**) WT mice. (*B*) TSLP protein in skin lysates after treatment with inhibitors. (*C*) TSLP mRNA. (**D-F**) WT vs PAR-2-KO mice. (*D*) TSLP protein after c48/80. (*E*) TSLP mRNA expression. (f) TSLP protein upon stimulation of mSEs by mMCP6 for 12 hours. TSLP was quantified by (*B, D,* and *F*) ELISA and (*C* and *E*) qPCR. Means ± SEMs of 6-12 experiments/mice, **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001.

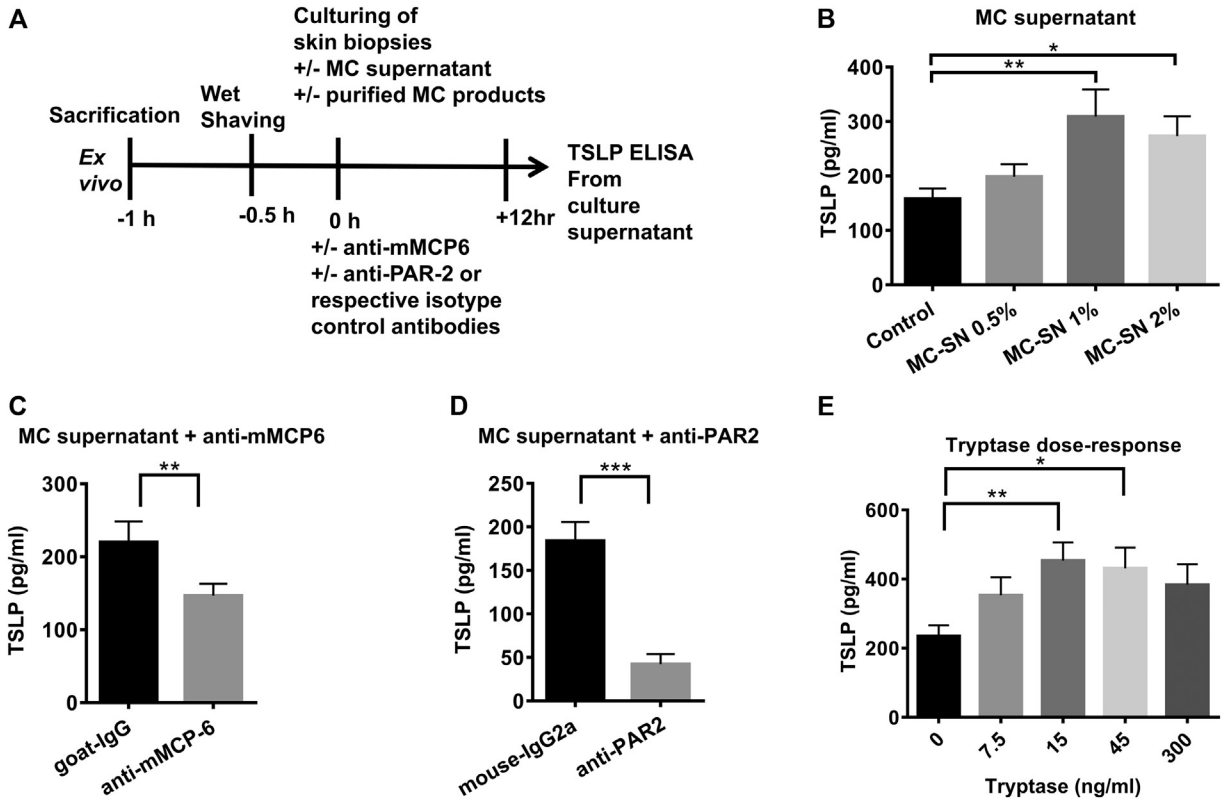

FIG 3. MC secretory products and exogenous tryptase stimulate TSLP via PAR-2 activation. (**A**) Scheme of the *ex vivo* model. (**B-E**) mSEs were treated for 12 hours with (*B*) MC-SN, (*C*) MC-SN in the presence of neutralizing anti-mMCP6 (or goat-IgG as control), (*D*) MC-SN in the presence of neutralizing anti–PAR-2 (or mouse-IgG2a), and (*E*) recombinant tryptase. TSLP was determined by ELISA. Means ± SEMs of 7-22 experiments, $*P < .05$, $**P < .01$, $***P < .001$. *SN,* Supernatant.

than after stimulation), while stimulated MCs also secrete multiple other entities (eg, eicosanoid mediators), we used baseline supernatants for this purpose (Fig 3, *A*). The MC secretome indeed triggered TSLP in murine skin whereby an optimum was reached at 1% (vol/vol) (Fig 3, *B*). Pretreating the supernatant with a neutralizing anti-mMCP6 antibody before administration to the skin attenuated TSLP generation (Fig 3, *C*). In a complementary approach, an anti–PAR-2 neutralizing antibody applied to the explant before the supernatant curbed TSLP production (Fig 3, *D*). Finally, recombinant mMCP6 elicited TSLP (Fig 3, *E*). Histamine, another crucial mediator of the MC secretome, was without effect over a broad range of concentrations (see Fig E3, *A* and *B,* in the Online Repository available at www.jacionline.org). Conversely, histamine at 10 μmol potently elicited IL-6, IL-8, and stem cell factor (SCF) in human KCs, demonstrating effectiveness at the level of other cytokines (Fig E3, *C-F*). In addition, chymase, another MC-selective protease,[38-41] did not stimulate TSLP production—not alone, and not in cooperation with tryptase (Fig E3, *G*). Collectively, MC mediators can elicit TSLP, and the tryptase/PAR-2 axis plays an instrumental part in this response.

IL-1 and tryptase act in concert to activate TSLP in the skin

There is remarkable cross talk between PAR-2 and the IL-1 system in KCs after activation of PAR-2 with synthetic agonists.[14,42] To ascertain validity under natural, protease-driven PAR-2 activation, we stimulated skin samples with mMCP6, IL-1α, and both combined. Indeed, together, the stimuli enhanced TSLP secretion vis-à-vis each individual stimulus (Fig 4). We asked whether tryptase operates indirectly by inducing IL-1, which subsequently elicits TSLP. However, IL-1RA only interfered with TSLP generation when IL-1 was present, but not with baseline or tryptase-driven production (Fig 4, *B*). The outcome was comparable when tryptase was substituted by a synthetic PAR-2 agonist (Fig E2, *B*). Together, tryptase works independently of, but can cooperate with, the proinflammatory IL-1 network in the skin environment.

Exogenous tryptase rescues TSLP responses in MC-deficient mice

Having found that MC-deficient mice were unable to mount a TSLP response upon stimulation with c48/80 (Fig 1), we asked whether exogenous provision of tryptase may bypass the dependency on MCs. In fact, while stimulation with mMCP6 had no additional effect when administered together with c48/80 in mSEs from MC-sufficient mice, the protease could fully reestablish the response in mSEs from MC-deficient mice (Fig 5). Of note, MC-deficient mice tended to show lower TSLP production even at baseline, potentially resulting from spontaneous (or neuropeptide-driven) tryptase release. We conclude that MCs essentially contribute to TSLP responses in murine skin chiefly through the release of tryptase.

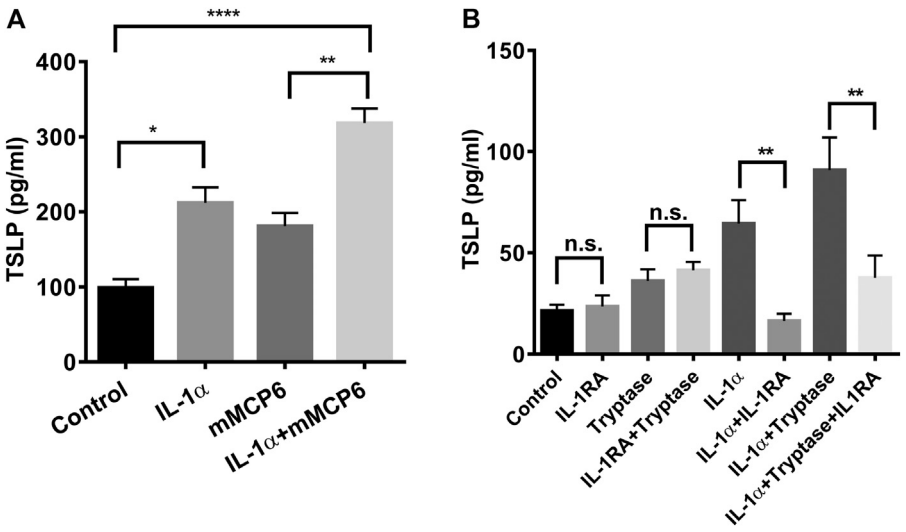

FIG 4. IL-1 and tryptase act in concert to elicit TSLP in the skin. (**A**) mSEs were stimulated (or not) with IL-1α and/or mMCP6 for 12 hours. TSLP was quantified in the supernatants using ELISA. Means ± SEMs of 12 experiments. (**B**) hKCs were stimulated with IL-1α and/or human tryptase for 24 hours in the presence or absence of IL-1RA. Means ± SEMs of 3 experiments, $*P < .05$, $**P < .01$, $****P < .0001$.

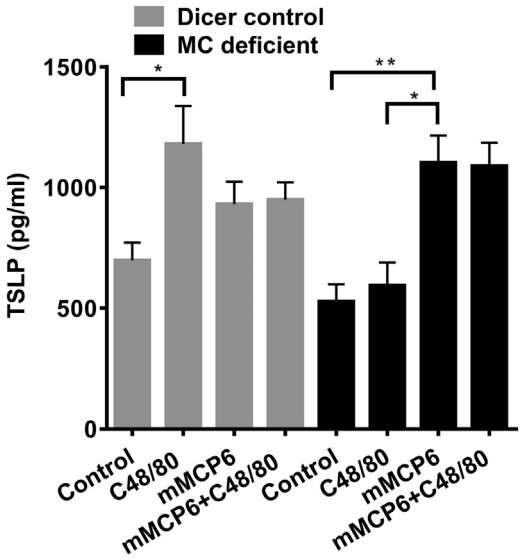

FIG 5. Exogenous tryptase rescues TSLP responses in MC-deficient mice. mSEs from Dicer control and MC-deficient mice were stimulated with c48/80 in the presence or absence of exogenous mMCP6 or kept without stimulation. TSLP in culture supernatants was quantified by ELISA. Means ± SEMs of 10 independent experiments, $*P < .05$, $**P < .01$.

Human skin MCs instruct human KCs to produce TSLP in a tryptase-dependent manner

By using *ex vivo* skin MCs and primary human KCs, we finally examined whether the connection identified among MCs, tryptase, KCs, and TSLP identified in mice would also apply to humans. In preliminary experiments, KCs were treated with MC supernatants or lysates over a wide range of concentrations (0.05-10% vol/vol). The highest effectiveness was found at 0.05-0.1% (lysate) and around 0.5% (supernatant) (Fig 6, *A*). Lysates or supernatants did not contain measurable TSLP in the concentrations used (data not shown). The tryptase inhibitor nafamostat mesylate attenuated TSLP production nearly to baseline (Fig 6, *B*). In another approach, MC lysate was used, from which tryptase had been specifically removed by IP, eliminating responsiveness of KCs to MC biomolecules (Fig 6, *C* and *D*). Additionally, we stimulated KCs with human tryptase in the presence of IL-1α. While tryptase or IL-1α alone were able to elicit TSLP, costimulation by both resulted in more pronounced responses (Fig 6, *E*).

Together, human skin MCs activate TSLP in human KCs on account of their major granule protease tryptase.

DISCUSSION

Considering the well-established link between MCs and skin pathologies like AD, this study investigated whether MCs communicate with KCs to instruct epithelial cytokine production. We report that the selective activation of MCs in murine skin indeed elicits TSLP in the epidermis, suggesting the existence of an MC/KC/TSLP axis.

To achieve selective activation of MCs in the skin, c48/80 was used, the canonical agonist of MRGPRX2/Mrgprb2, which has come to the forefront of scientific scrutiny as the mediator of pseudoallergic/neurogenic MC responses.[34,36,43,44] Evidence is accumulating that MRGPRX2 acts as a critical determinant in diseases like urticaria, allergic contact dermatitis, and AD.[4,35,45] In the present study, c48/80 stimulation elicited TSLP in skin explants in a strictly MC-dependent fashion because only MC-sufficient mice but not their MC-deficient counterparts exhibited responsiveness. In our *ex vivo* setting, baseline TSLP expression was relatively high, resulting from trauma on explant preparation, as described previously.[13,14,46] In fact, TSLP sensitively marks any kind of deviation from homeostasis, including microbial/inflammatory insults or physical or chemical irritation.[13,14] Inducibility of TSLP by c48/80 was reproduced *in vivo,* where it was even more pronounced as a result of the lower production in the control (increase by 3.2-fold *in vivo* vs 1.7-fold *ex vivo*). Importantly, *in vivo* responses perfectly reproduced the dependence on MCs.

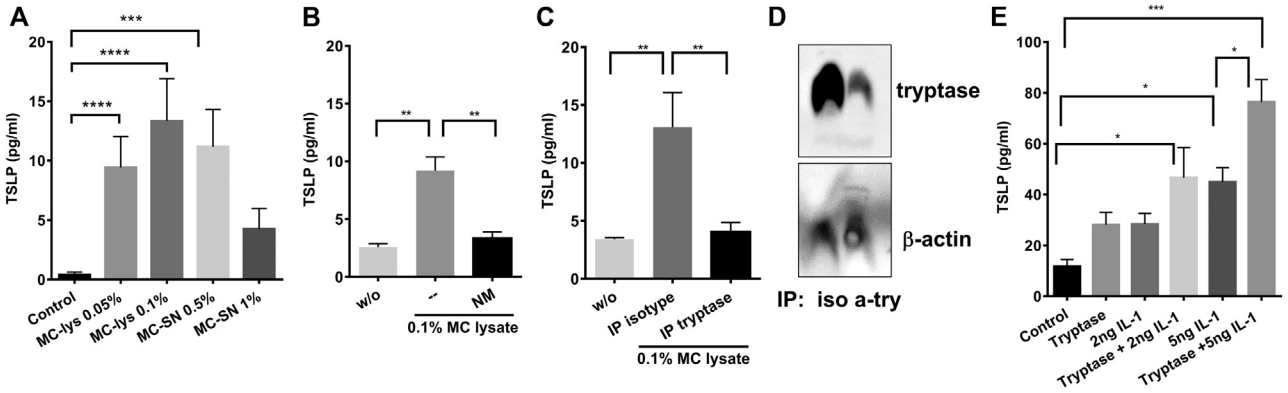

FIG 6. Human skin MCs instruct human KCs to produce TSLP through tryptase. KCs were incubated with (**A**) MC lysate (*lys*) or supernatant (*SN*), (**B**) MC lysate in the presence or absence of nafamostat mesylate (*NM*), and (**C**) after elimination of tryptase by IP. (**D**) Immunoblot showing reduction of tryptase after IP. (**E**) KCs were stimulated with tryptase and/or IL-1α. TSLP in culture supernatants was quantified by ELISA. Means ± SEMs of 8-15 independent experiments, $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$.

We investigated the mechanisms by which MCs instruct KCs to upregulate TSLP. The rapidness with which TSLP protein was detectable in the skin upon injection of c48/80 indicated that preformed MC mediators were involved in this process. In addition to biogenic amines (especially histamine), MCs are rich sources of prefabricated neutral proteases (tryptase, chymase, and carboxypeptidase A), with tryptase being the most abundant entity.[41,47,48] Indeed, we found that interference with tryptase, but not antagonization of histamine receptors, inhibited MC-dependent TSLP induction. Chymase was likewise without effect. Because tryptase is a potent activator of PAR-2[37,47,49,50] and PAR-2 has been associated with TSLP responses,[14,24,51,52] we used PAR-2-KO mice and could prove its involvement in TSLP induction. Moreover, PAR-2 deficiency protected from tryptase-mediated TSLP generation. The latter finding provides evidence that tryptase acts by activation of PAR-2 in this setting without a major impact of other substrates.[24] In a complementary strategy, murine skin was exposed to MC supernatants containing the spontaneously secreted preformed mediators (released, eg, by piecemeal degranulation[53]), whereby 1% (vol/vol) of supernatant was already sufficient to elicit a maximum response. Importantly, TSLP induction was sensitive to neutralizing antibodies against mMCP6 or PAR-2. Correspondingly, purified tryptase substituted for MC supernatant, verifying that tryptase was the active component in the complex mixture of MC-derived biomolecules. Conversely, histamine had no direct effect on TSLP production in accordance with the lacking effect of histamine receptor antagonists. However, histamine may contribute to TSLP generation indirectly via eliciting other cytokines (Fig E3). This indirect route seems particularly relevant in the absence of PAR-2. In fact, both IL-1β and TNF-α were strongly enhanced by c48/80 in PAR-2–deficient mice (see Fig E4, *B* and *C,* in the Online Repository at www.jacionline.org), potentially explaining why PAR-2-KO animals produce TSLP upon c48/80 injection (Fig 2, *D* and *E*) even though they are unable to respond to tryptase. The main direct route, which is chiefly operative in PAR-2–intact mice, depends on tryptase, however.

Interestingly, higher concentrations of MC lysates or tryptase resulted in lower rather than higher TSLP responses. In fact, TSLP is susceptible to cleavage by MC-derived tryptase, and even a particularly relevant target among a plethora of cytokines tested.[54,55] Our findings imply that at low concentrations PAR-2 activation prevails but that this effect gets saturated, while higher amounts of tryptase are required to cleave TSLP, explaining the bell-shaped dose–response curve.

Purified tryptase (mMCP6) was able to bypass MC deficiency and to fully restore TSLP production. In contrast, exogenous tryptase had no additional effect in MC-sufficient surroundings, indicating that the intact MC population of the skin already achieved a maximum response. In a translational approach we confirmed that biomolecules from human MCs were potent inducers of TSLP in human KCs, where tryptase was again revealed as the active principle.

As indicated above, PAR-2–deficient mice still responded to c48/80, albeit significantly less than WT mice. Nevertheless, this raises the question of whether other mechanisms combine with the tryptase/PAR-2 axis to further enhance TSLP induction, even though in most settings of intact PAR-2 signaling (Fig 2, *B* and *C;* Fig 3; and Fig 6, *B-D*), interference with tryptase was highly effective at abrogating the TSLP response. Evidently induction by c48/80 was MC dependent even if not fully tryptase dependent (cf Fig 2 and Fig 1). Accordingly, direct treatment of KCs with c48/80 did not stimulate TSLP (data not shown). Importantly, PAR-2–deficient mice were more receptive to proinflammatory cytokines like IL-1 (Fig E4, *A*), and they also produced more IL-1β and TNF-α in the skin upon c48/80 injection, indicating that MC stimulation in the skin elicits other inflammatory mediators that further feed into TSLP production. Considering that histamine can induce various cytokines in KCs, it is possible that part of this response is driven by histamine. The reason for why PAR-2-KO mice produce and respond to other inflammatory stimuli more vigorously potentially results from the missing "second stimulus" through PAR-2, eliciting compensatory upregulation of other pathways such as components pertaining to the IL-1 system and perhaps histamine. This scenario would be compatible with MC dependence but tryptase independence of a fraction of the response, and this fraction seems considerably more important when PAR-2 is missing, as demonstrated in Fig E4.

We reported that PAR-2 activation by a synthetic ligand cooperates with IL-1, resulting in significant TSLP generation even at low IL-1 concentrations.[14] We were interested in whether tryptase could substitute for the agonistic peptide. Additive

effects of tryptase and IL-1 were detected in mouse skin and human KCs alike, substantiating the notion that mutual potentiation was preserved across species. Our findings imply that in the presence of subtle inflammation, MC-derived tryptase may tip the scales toward manifest inflammation by elevating TSLP above the threshold required for biologically meaningful responses in dendritic cells, eosinophils, basophils, and MCs.[56,57]

The significance of tryptase to AD pathology is underscored by several lines of evidence. In fact, both the protease itself and its receptor, PAR-2, are upregulated in AD skin and correlate with pruritus.[58] Interestingly, AD itch, a debilitating hallmark of the disease, has long been known to proceed in a mostly histamine-independent fashion.[4,17,59-62] In accordance, MC degranulation and PAR-2 activation were associated with scratching in a house dust mite–induced AD model, whereby MC activation also induced TSLP by an unknown mechanism,[63] while another study uncovered that tryptase but not histamine forms the essential constituent in dermatitis models of Mrgprb2-dependent MC activation.[64] Degranulating MCs were found adjacent to activated neurons in yet another model, and house dust mite extracts induced $TRPV1^{+}Tac1^{+}$ nociceptors to release neuropeptides that subsequently activated skin MCs via Mrgprb2.[65] Our data further extend these concepts: they indicate that secreted tryptase will not only directly induce itch in sensory neurons but also do so indirectly via tryptase-activated TSLP in KCs, further feeding into the itch program.[51] Evidence is also accumulating that TSLP may function as a bridge between KCs and MCs in the skin micromilieu by acting in a feed-forward loop. While MC activation *in situ* will induce epidermal TSLP by a tryptase-dependent mechanism, as demonstrated herein, TSLP can subsequently act on skin MCs to enhance survival[66] and foster their secretory competence at the level of cytokines,[39] but importantly also at the level of degranulation, thereby further increasing tryptase secretion.[67] Interestingly, only the MRGPRX2-dependent route is supported by TSLP in skin MCs, while Fc epsilon receptor 1–dependent stimulation is not significantly altered.[67] Combined, these findings indicate that MCs are important targets of the cytokine modulation they themselves provoke. This is corroborated by the abundant expression of the TSLP receptor (gene *CRLF2*) in MCs, and also when viewed across the comprehensive FANTOM5 (Functional Annotation of the Mammalian Genome 5) body-wide atlas.[66,68] The regulatory network connecting KCs, MCs, tryptase, and TSLP may jointly and gradually enhance inflammation in the skin environment, making counterregulation even more necessary. One of the built-in off switches to keep the loop at bay lies in the inherent ability of tryptase to not only induce but also degrade TSLP,[26,54,55] as is also underlined by our bell-shaped dose–response curves. Tryptase-driven TSLP production may not be limited to the skin but may also occur in the respiratory tract because tryptase is universally present in MCs.[28]

Collectively, our findings reveal that the communication between MCs and KCs in the skin micromilieu may be stronger than hitherto suspected and encompass tryptase as an important constituent. Because MCs are the exclusive source of tryptase, our results strengthen the significance of MCs in the production of TSLP, providing a rational link between MC abundance in inflammatory disorders, such as AD, and overproduction of TSLP. Targeting this cross talk may help develop novel therapeutics to treat these pathologies. Indeed, an allosteric antibody against human tryptase has recently been developed and proposed as a treatment modality for severe asthma.[69] It may be worth addressing whether interference with tryptase may constitute a novel approach in the treatment of AD.

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Clinical implications: Awareness of the cross talk between MCs and KCs may permit improved management of skin disorders—for example, by selective targeting of tryptase.

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METHODS

Reagents

Recombinant human and murine IL-1α was purchased from Miltenyi Biotec (Bergisch-Gladbach, Germany). Anti–PAR-2 antibody (SAM-11) was from Santa Cruz Biotechnology (Heidelberg, Germany); mouse IgG_2a isotype control antibody and recombinant human IL-1 receptor antagonist were from Immunotools (Cloppenburg, Germany). mMCP6, goat anti-mouse MCP6 antibody, and goat-IgG control antibody were from R&D Systems (Wiesbaden-Nordenstadt, Germany). Histamine, JNJ maleate, desloratadine, c48/80, and recombinant human chymase were from Sigma-Aldrich (Steinheim, Germany). Recombinant human skin β tryptase was from Promega (Madison, Wisc). The PAR-2 agonist 2-furoyl-LIGRLO-amide as well as the SLIGRL-NH_2 were from Tocris Bioscience (Bristol, United Kingdom).

RNA isolation, reverse transcription, and qPCR

The NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) was used for RNA isolation. Skin samples were homogenized by Precellys Homogenisation (Peqlab) in RA1 buffer, followed by proteinase K (Macherey-Nagel) digestion for 15 minutes at 55°C. All steps were performed according to the manufacturer's protocol with an additional step of DNase (Macherey-Nagel) digestion for 15 minutes at room temperature.

Then RNA was reverse transcribed with TaqMan reverse transcription reagents (Applied Biosystems, Darmstadt, Germany), and qPCR was performed with the rotor gene SYBR Green PCR kit on the rotor gene thermocycler (both from Qiagen, Hilden, Germany) according to the manufacturer's protocol. Oligonucleotide primers (synthesized by Tib Molbiol, Berlin, Germany) and their annealing temperatures are specified in Table E1. Expression levels of target genes were quantified relative to the expression of the reference gene hypoxanthine–guanine phosphoribosyltransferase (HPRT) using the 2-$\Delta\Delta C_t$ method. The relative expression values (ie, the gene of interest normalized to HPRT for each cDNA individually, with both genes run in parallel) were used to create the respective figures.

Enzyme-linked immunosorbent assay

To detect mouse and human TSLP, ELISA kits were purchased from R&D Systems and eBioscience (both Frankfurt, Germany), respectively. For the detection of human IL-8, an ELISA kit was purchased from Invitrogen (Cloppenburg, Germany). Quantification was performed according to the manufacturer's instructions. The analytical sensitivity for mouse TSLP, human TSLP, and human IL-8 ELISA, respectively, was 6.3, 3, and 5 pg/mL, respectively.

Skin explant treatment

Mice were anesthetized and humanely killed, followed by wet shaving of the abdominal region. Skin biopsy samples (5 mm^2) were taken and incubated in 150 μL of hydrocortisone hemisuccinate–free medium for 12 hours with or without c48/80 (100 μg/mL) with and without recombinant mMCP6 (10 ng/mL). Alternatively, mSEs were stimulated with recombinant human tryptase (7.5, 15, 45, and 300 ng/mL), histamine (0.2, 2, and 10 μmol), recombinant mouse IL-1α (10 ng/mL), or 2-furoyl-LIGRLO-amide (500 nmol). ELISA was performed on the resulting supernatants.

For inhibition studies, skin explants were stimulated with MC supernatant either in the presence or absence of anti-mMCP6 or anti–PAR-2 antibodies (goat-IgG and $mIgG_2a$ served as isotype controls, respectively).

IF staining and immunohistochemistry

Mouse skin biopsy samples were fixed in 4% paraformaldehyde at 4°C for 1 hour, cryoprotected by 15% sucrose solution, embedded in optimal cutting temperature compound, and cryosectioned to 10 μm thick. All specimens were then processed for IF using standard procedures.[E1]

Primary rabbit anti-TSLP (1:300, Invitrogen, Karlsruhe, Germany) and secondary goat anti-rabbit Alexa Fluor 568 (1:500, Invitrogen) antibodies were used for IF staining. To visualize the skin morphology, hematoxylin and eosin staining was performed following standard procedures.

Removal of tryptase by IP

For IP, MCs were lysed in phosphate-buffered saline containing protease inhibitors using freeze–thaw cycles, and the insoluble fraction was pelleted. The soluble lysates were incubated with anti-tryptase polyclonal antibody (25 μg/mL) (polyclonal goat-IgG, R&D Systems); goat-IgG served as control. Protein G beads (Santa Cruz Biotechnology) were added and incubated overnight at 4°C on a rotary shaker. The precleared lysates were subjected to the same IP protocol for 2 more times. After the final IP, MC lysates were used for KC stimulation, and KC culture supernatants collected after 24 hours for TSLP measurement by ELISA. MC lysates were also tested for TSLP, but concentration was below detection.

To verify tryptase elimination, MC lysates were resuspended in sample buffer, loaded on 10% polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane. The membrane was probed with anti-tryptase antibody (polyclonal goat-IgG, R&D Systems, 1 μg/mL), followed by detection using a conjugated horseradish peroxidase (Santa Cruz Biotechnology) secondary antibody.

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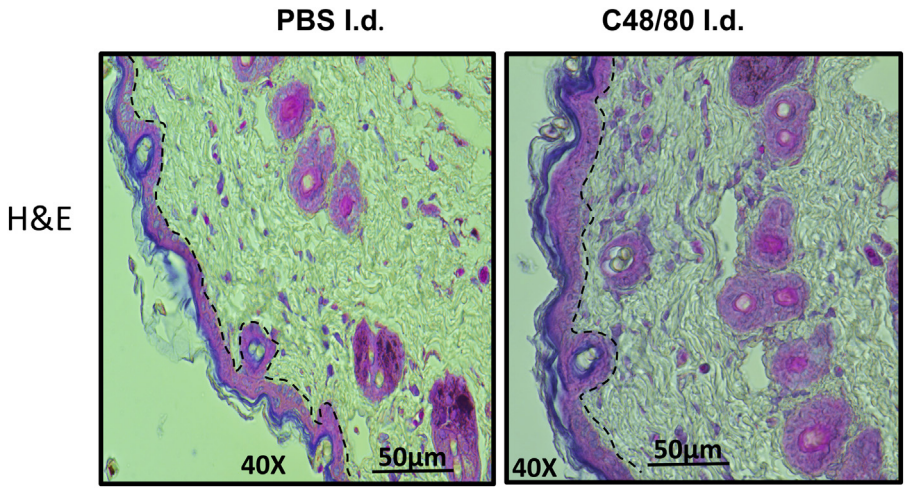

FIG E1. Representative skin sections (hematoxylin and eosin, original magnification 40×) corresponding to Fig 1, *E*.

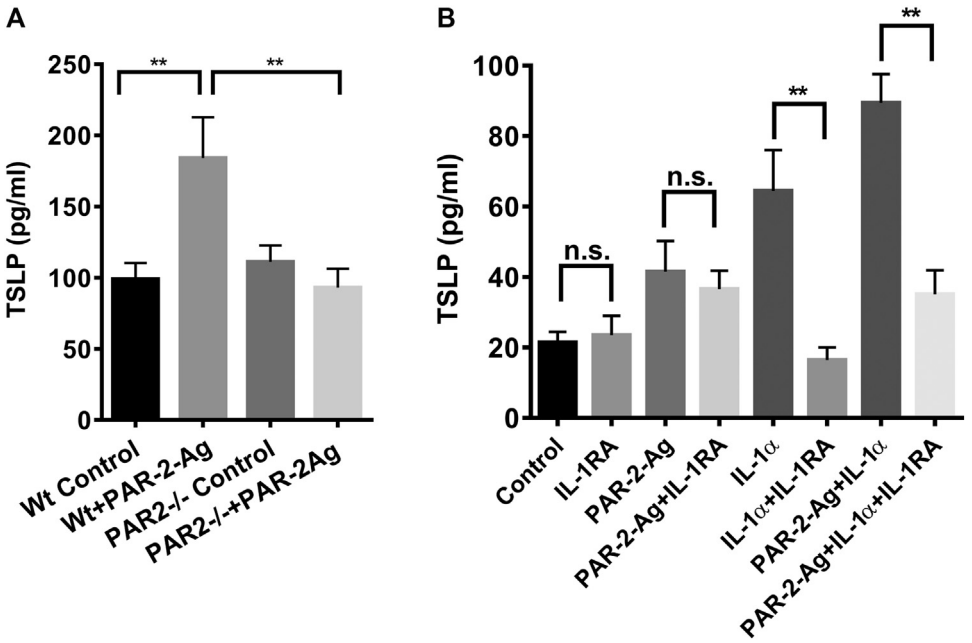

FIG E2. PAR-2 activation by a synthetic agonist activates TSLP. (**A**) mSEs from WT vs PAR-2-KO mice were incubated with the selective PAR-2 agonist 2-furoyl-LIGRLO-amide (2-Fly) for 12 hours. Means ± SEMs of 6 experiments. (**B**) hKCs were stimulated with IL-1RA (100 ng/mL), PAR-2 agonist SLIGRL-NH_2 (100 μmol), and IL-1α (10 ng/mL) alone or in combination for 24 hours. Untreated hKCs served as control. Means ± SEMs of 3 experiments. TSLP was quantified in the culture supernatants using ELISA kits for murine (*A*) and human (*B*) TSLP, respectively. $^{**}P < .01$.

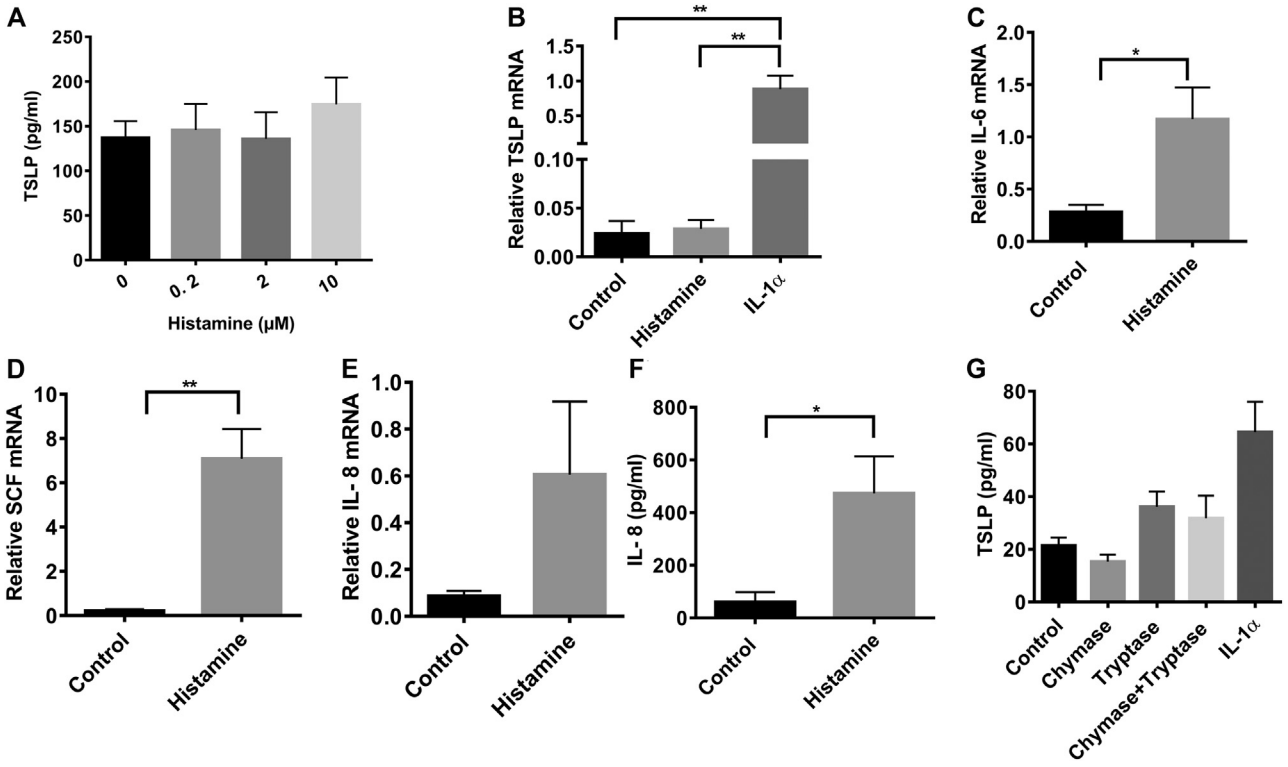

FIG E3. Histamine and chymase do not alter TSLP production, but histamine elicits other cytokines. (**A**) mSEs were incubated with different concentrations of histamine for 12 hours, and murine TSLP was measured by ELISA. Means ± SEMs of 12 independent experiments. (**B-E**) hKCs were stimulated with histamine at 10 μmol for 2.5 hours and the mRNA was collected for qPCR. Expression of the target gene was normalized to HPRT. (*B*) TSLP; IL-1α at 10 ng/mL served as positive control. (*C*) IL-6. (*D*) SCF. (*E*) IL-8. (**F**) hKCs were stimulated with histamine at 10 μmol for 24 hours and IL-8 quantified by ELISA. (**G**) hKCs were stimulated with chymase (250 ng/mL), tryptase (15 ng/mL), or both for 24 hours. IL-1α at 10 ng/mL served as positive control. IL-8 protein (*F*) and TSLP protein (*G*) were measured by ELISA. (*B-G*) Means ± SEMs of 3 independent experiments. **P* < .05, ***P* < .01.

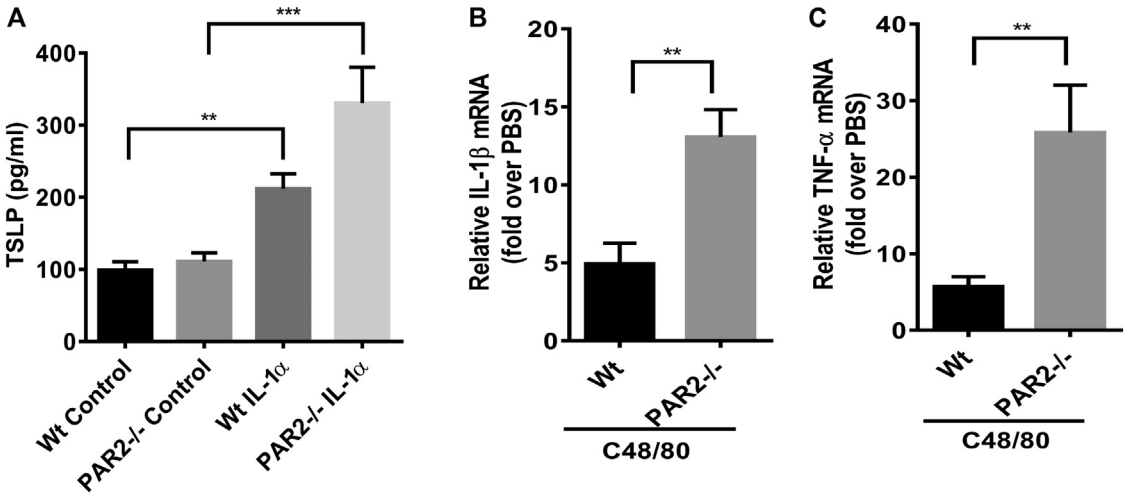

FIG E4. PAR-2 deficiency confers enhanced responsiveness to IL-1 and c48/80. (**A**) mSEs from WT and PAR-2-KO mice were incubated for 12 hours in the presence or absence of IL-1α. TSLP was quantified in the supernatants by ELISA. mRNA expression of (**B**) IL-1β and (**C**) TNF-α in the skin of WT and PAR-2-KO mice after intradermal injection of c48/80 (see Fig 2, *E*). mRNA expression was quantified by qPCR. Means ± SEMs of 6 independent experiments. $*P < .05$, $**P < .01$, $***P < .01$.

TABLE E1. Primers used for qPCR in murine and human studies

Gene	Primers (5′-3′)		Annealing temperature (°C)
	Forward	Reverse	
mTslp	AGAGAAGCCCTCAATGACCA	GGACTTCTTGTGCCATTTCC	61
mHprt	CGTCGTGATTAGCGATGATG	AATCCAGCAGGTCAGCAAAG	60
hTSLP	TCGCTCGCCAAAGAAATGTT	TCGCTCGCCAAAGAAATGTT	65
hIL-6	ATGTAGCCGCCCCACACAGA	CATCCATCTTTTTCAGCCAT	62
hIL-8	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAACTTCTC	62
hSCF	GCGTGGACTATCTGCCGCCG	AGCGCTGCGATCCAGCACAAA	62
hHPRT	ATCAGACTGAAGAGCTATTG TAATGACCA	TGGCTTATATCCAACACTTCGTG	64