

Cytokine milieu modulates release of thymic stromal lymphopoietin from human keratinocytes stimulated with double-stranded RNA

Hirokazu Kinoshita, MD,^{a,b} Toshiro Takai, PhD,^a Tuan Anh Le, MD,^{a,b} Seiji Kamijo, PhD,^a Xiao Ling Wang, MD,^a Hiroko Ushio, DVM, PhD,^a Mutsuko Hara, BSc,^a Junko Kawasaki, MD,^{a,b} Anh Tuan Vu, MD,^{a,b} Takasuke Ogawa, MD,^{a,b} Hendra Gunawan, MD, PhD,^{a,b} Shigaku Ikeda, MD, PhD,^b Ko Okumura, MD, PhD,^{a,c} and Hideoki Ogawa, MD, PhD^a Tokyo, Japan

Background: Thymic stromal lymphopoietin (TSLP) plays a key role in allergic diseases, such as atopic dermatitis (AD) and asthma. TSLP is highly expressed by keratinocytes in skin lesions of patients with AD, but environmental triggers for its release from keratinocytes with endogenous factors are not well understood. Patients with AD, in whom allergic sensitization is already established, are susceptible to viral dissemination. **Objectives:** We investigated TSLP's release from primary human keratinocytes stimulated with a Toll-like receptor (TLR) 3 ligand, polyinosinic-polycytidylic acid, which mimics viral double-stranded RNA (dsRNA), and its modulation by cytokines.

Methods: Primary human keratinocytes were stimulated with TLR ligands, cytokines, or both. TSLP released into culture supernatants was measured by means of ELISA.

Results: Stimulation of keratinocytes with dsRNA induced release of TSLP and upregulated gene expression of TSLP and other cytokines and chemokines. The release of TSLP was enhanced by the addition of IL-4, IL-13, and/or TNF- α . With or without the T_H2/TNF cytokines, the dsRNA-induced release of TSLP was upregulated by IFN- α and IFN- β and suppressed by IFN- γ , TGF- β , or IL-17.

Conclusions: The effect of the TLR3 ligand on keratinocytes suggests contribution of viral dsRNA to skin inflammations under the influence of a cytokine milieu. The results imply that viral dsRNA and a T_H2 cytokine milieu might promote T_H2-type inflammation through an induction of TSLP expression, suggesting that a vicious cycle exists between AD with T_H2-type inflammation and viral infections and a possible blockade of this cycle by other cytokine milieus provided by cells, such as T_H1, regulatory T, and T_H17 cells. (J Allergy Clin Immunol 2008;122:1000-1008.)

Key words: Thymic stromal lymphopoietin, keratinocyte, double-stranded RNA, cytokine milieu, atopic dermatitis, viral exposure, viral infection, vicious cycle, promotion and blockade

Recent findings indicate that thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine, plays a key role in allergic inflammation.^{1,2} TSLP is highly expressed by keratinocytes in skin lesions of patients with atopic dermatitis (AD)³ and airway epithelial cells of patients with asthma.⁴ TSLP was found in keratinocytes of skin lesions of patients with acute and chronic AD but not in their nonlesional skin and lesions of patients with nickel-induced allergy contact dermatitis or cutaneous lupus erythematosus.³ TSLP-activated dendritic cells (DCs) secrete T_H2-recruiting chemokines but not IL-12 and induce naive T cells to differentiate into inflammatory T_H2 cells, producing IL-4, IL-5, IL-13, and TNF- α through OX40 ligand.^{1,2,5} TSLP similarly activates epidermal Langerhans cells, a subset of DCs.⁶ The TSLP DCs can induce allergen-specific T_H2 memory cells to undergo homeostatic expansion and further T_H2 polarization and to mediate recall responses.⁷ In support of the findings in human subjects, mice with keratinocyte- or lung-specific overexpression of TSLP have an AD- or asthma-like phenotype,^{8,9} and mice lacking the TSLP receptor have considerably attenuated disease in asthma models.^{9,10} TSLP can also directly act on human mast cells synergistically with IL-1 and TNF- α to produce IL-5 and IL-13¹¹ and on human CD4⁺ T cells activated with T-cell receptor stimulation to markedly proliferate.¹² Thus TSLP represents a critical factor linking responses at interfaces between the body and environment to allergic type 2 immune responses.

In human primary airway epithelial cells, the release of TSLP *in vitro* is induced on stimulation with polyinosinic-polycytidylic acid (poly I:C), mimicking viral double-stranded RNA (dsRNA), and the proinflammatory cytokines TNF- α and IL-1, mimicking an inflammatory microenvironment.¹¹ The T_H2 cytokines IL-4 or IL-13, mimicking an allergic microenvironment, upregulate the poly I:C-induced release of TSLP and the level of TSLP mRNA synergistically with rhinovirus infection,¹³ suggesting that viral infections in a T_H2 cytokine milieu amplify airway allergic reactions. Poly I:C also induces TSLP's release from human tonsillar epithelial cells.¹⁴

In human keratinocytes the environmental factors triggering TSLP's release and its modulation with endogenous factors are not well investigated, although Bogiatzi et al¹⁵ have recently reported that proinflammatory and T_H2 cytokines act synergistically to induce the release of TSLP from human skin explants

From ^athe Atopy (Allergy) Research Center, ^bthe Department of Dermatology, and ^cthe Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan. Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

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Reprint requests: Toshiro Takai, PhD, Atopy (Allergy) Research Center, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan.

E-mail: t-takai@juntendo.ac.jp.

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Abbreviations used

AD:	Atopic dermatitis
DC:	Dendritic cell
dsRNA:	Double-stranded RNA
Pam ₃ CSK ₄ :	(S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys ₄ -OH, 3HCl
poly I:C:	Polyinosinic-polycytidylic acid
TLR:	Toll-like receptor
Treg:	Regulatory T
TSLP:	Thymic stromal lymphopoietin

obtained from healthy donors. Patients with AD tend to have widespread disseminated viral infections.¹⁶ Disturbed skin barrier¹⁷⁻¹⁹ and decreased expression of LL-37,²⁰ which has innate immune function to control viral replication,²¹ in AD skin are considered to provide easier access for the virus and permit its replication, respectively. In the present study we analyzed TSLP's release from primary human keratinocytes stimulated with dsRNA in the absence or presence of cytokines.

METHODS**Reagents**

The following recombinant human cytokines and concentrations were used to stimulate keratinocytes: 20 ng/mL TNF- α , 100 ng/mL IL-4, 100 ng/mL IL-13, 100 ng/mL IFN- γ , 100 ng/mL IL-10, 10 ng/mL TGF- β , 100 ng/mL IL-17, 100 ng/mL IL-22 (R&D Systems, Minneapolis, Minn), 10 ng/mL (2000 U/mL) IFN- α , and 100 ng/mL (2000 U/mL) IFN- β (PeproTech, London, United Kingdom). The following Toll-like receptor (TLR) ligands and concentrations were used to stimulate keratinocytes: 0.1 to 100 μ g/mL poly I:C; 5 μ g/mL (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys₄-OH, 3HCl (Pam₃CSK₄; Calbiochem, San Diego, Calif); 20 μ g/mL peptidoglycan from *Staphylococcus aureus*; 20 μ g/mL LPS from *Escherichia coli* serotype 0111:B4 (Sigma, St Louis, Mo); 20 ng/mL flagellin purified from *Salmonella typhimurium* strain 14028 (Alexis Biochemicals, San Diego, Calif); 20 μ g/mL loxoribine; 10 μ g/mL CL097 (InvivoGen, San Diego, Calif); and 5 μ mol/L human CpG (Hycult biotechnology, Uden, The Netherlands).

Cell culture and stimulation of keratinocytes

Primary human keratinocytes (Cascade Biologics, Portland, Ore) were cultured in EpiLife KG2 (Kurabo, Osaka, Japan) supplemented with 0.1 ng/mL epidermal growth factor, 10 μ g/mL insulin, 0.5 μ g/mL hydrocortisone, 50 μ g/mL gentamycin, 50 ng/mL amphotericin B, and 0.4% vol/vol bovine brain pituitary extract. Cells were seeded at 1×10^4 cells/well in flat-bottomed, 96-well microculture plates and cultured until they reached 80% confluence, and then the medium was renewed. After further cultivation for 24 hours, cells were stimulated with TLR ligands, cytokines, or both in experiments, except for quantitative PCR for gene expression at 4 and 8 hours, in which the medium used for the stimulation and 24-hour cultivation before the stimulation did not contain hydrocortisone.

ELISA

Concentrations of TSLP and IL-8 protein were measured by using diluted (1:2 for TSLP and less than 1:20 for IL-8) culture supernatants collected at 48 hours after the stimulation with ELISA kits (DuoSet, R&D Systems). In this study the minimum detection limits for TSLP and IL-8 in the supernatants were 7.8 and 156 pg/mL, respectively.

Real-time quantitative PCR

Total RNA was extracted from cells with RNeasy Mini (GE Healthcare, Buckinghamshire, United Kingdom) and treated with DNase I (GE

Healthcare) in Fig 1, C, and RNeasy Plus Micro Kit (QIAGEN, Hilden, Germany) in Fig 2. cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif) and random primers. Real-time quantitative PCR was performed with a TaqMan method with an ABI 7500 (Applied Biosystems, Piscataway, NJ). The mRNA level was normalized to the gene expression of glyceraldehyde-3-phosphate dehydrogenase in Fig 1, C, and β -actin in Fig 2.

Statistical analysis

An unpaired Student *t* test (2-tailed) or 1-way ANOVA with the Tukey multiple comparison test was used. *P* values of less than .05 were regarded as statistically significant. Data shown are the means $\pm$ SDs for 3 wells, except for Fig 2, and are representative of 3 independent experiments.

RESULTS**dsRNA-induced TSLP release from keratinocytes**

Primary human keratinocytes were treated with TLR ligands, including synthetic bacterial lipoprotein Pam₃CSK₄ (TLR2 ligand), peptidoglycan (TLR2 ligand), poly I:C (TLR3 ligand), LPS (TLR4 ligand), flagellin (TLR5 ligand), loxoribine (TLR7 ligand), CL-097 (TLR7/8 ligand), and CpG (TLR9 ligand), to test for a possible role of the regulation of TSLP's production by TLR ligands in keratinocytes. ELISA of the culture supernatants showed that keratinocytes treated with poly I:C, mimicking viral dsRNA, released detectable amounts of TSLP (Fig 1, A). The other ligands tested did not induce TSLP's release, whereas peptidoglycan and flagellin induced IL-8's release, although less effectively than poly I:C (Fig 1, B), which is consistent with other reports.^{22,23} The gene expression for *TSLP* mRNA was significantly upregulated by stimulation with poly I:C (Fig 1, C).

Stimulation of keratinocytes with poly I:C induced a more than 10-fold increase of gene expression of TSLP and other cytokines (TSLP, IFN- β , TNF- α , IL-6, and GM-CSF) and chemokines (CXCL8/IL-8, CXCL9/monokine induced by IFN- γ , CXCL10/IFN-inducible protein 10, CXCL11/IFN-inducible T-cell α chemoattractant, CCL2/monocyte chemoattractant protein 1, CCL5/RANTES, CCL20/macrophage inflammatory protein 3 α , and CCL22/macrophage-derived chemokine; Fig 2).

T_H2 cytokines and TNF- α upregulated dsRNA-induced release of TSLP from keratinocytes

TSLP is highly expressed by keratinocytes in skin lesions of patients with AD.³ AD skin is characterized by the overexpression of the T_H2 cytokines IL-4 and IL-13.^{24,25} To test whether a cytokine milieu that mimics the atopic environment modulates poly I:C-induced release of TSLP, we examined whether a combination of TNF- α together with the T_H2 cytokines IL-4 and IL-13 (T_H2/TNF cytokines) would enhance TSLP's release from keratinocytes (Fig 3, A-C, and Fig 4, A-C). The dose- and time-dependent poly I:C-induced release was markedly upregulated by costimulation with the T_H2/TNF cytokines (Fig 3, A-C). A low level of TSLP was detectable on stimulation with the T_H2/TNF cytokines without poly I:C (Fig 4, A), supporting a recent study with skin explants obtained from healthy donors.¹⁵ Poly I:C significantly induced TSLP's release and was most effective in the presence of both TNF- α and one of the 2 T_H2 cytokines IL-4 and IL-13 (Fig 4, B and C). Removing TNF- α or both T_H2 cytokines decreased the amount of TSLP released, suggesting that signaling pathways through receptors for TNF- α and the T_H2

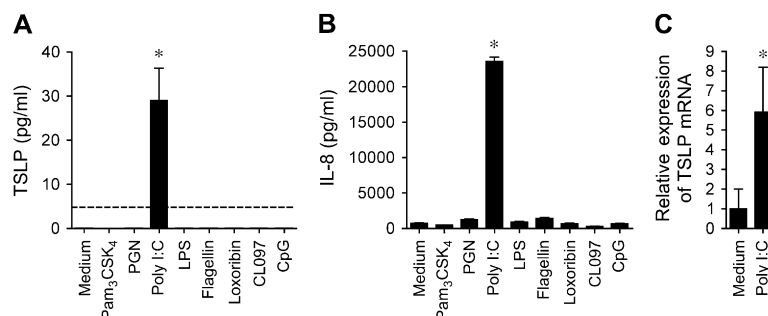


FIG 1. Poly I:C induced release of TSLP from keratinocytes: **A**, TSLP; **B**, IL-8. Broken line, Less than the minimum detection limit. PGN, Peptidoglycan. **C**, Expression of TSLP mRNA at 3 hours after stimulation was analyzed by means of quantitative real-time PCR and is presented as fold change relative to medium. * $P < .05$ versus medium by means of ANOVA (Fig 1, A and B) and t test (Fig 1, C).

cytokines synergistically upregulate the poly I:C-induced release. Removing one of the T_H2 cytokines had no effect (Fig 4, B and C), as supported by the finding that the IL-4 receptor α -chain is shared by receptors for IL-4 and IL-13 and is essential for activation of the central transcription factor signal transducer and activator of transcription 6.²⁶ TNF- α or poly I:C upregulated the release of IL-8, but IL-4 and IL-13 made little or no contribution to IL-8 levels (Fig 4, D-F).

Type I IFNs upregulated dsRNA-induced release of TSLP from keratinocytes

Because the type I IFNs IFN- α and IFN- β enhance the gene expression of TLR3 in keratinocytes,²⁷ we examined whether the type I IFNs enhance TSLP's release from keratinocytes (Fig 5, A-C). Addition of the type I IFNs enhanced the poly I:C-induced release and further upregulated it in the presence of the T_H2 /TNF cytokines (Fig 5, B and C). Without poly I:C, a combination of both type I IFNs promoted the T_H2 /TNF-induced release of TSLP (Fig 5, A). The type I IFNs upregulated the release of IL-8 to a lesser extent (Fig 5, E and F), which is in contrast to their marked contribution to TSLP's release (Fig 5, A-C).

IFN- γ , TGF- β , and IL-17 suppressed dsRNA-induced release of TSLP from keratinocytes

Next we tested whether cytokine milieu, which could be provided by T_H1 cells, regulatory T (Treg) cells, or T_H17 cells, modulate the poly I:C-induced release of TSLP (Fig 6). IFN- γ , TGF- β , or IL-17 significantly suppressed the release in the absence and presence of the T_H2 /TNF cytokines (Fig 6, B and C). IL-10 and IL-22 had no or little effect on TSLP's release (data not shown). On the other hand, release of IL-8 was modulated differently by the cytokines tested and poly I:C (Fig 6, D-F).

DISCUSSION

The present study is composed of 4 steps, as follows. In step 1 we demonstrated that dsRNA stimulates the release of TSLP by keratinocytes and transcription of genes for TSLP and other cytokines and chemokines (Figs 1 and 2). In step 2 we examined whether a cytokine milieu that mimics the atopic environment (T_H2 /TNF cytokines) promotes the dsRNA-induced release of TSLP (Figs 3 and 4) in relation to the fact that patients with AD tend to have widespread disseminated viral infections.¹⁶ In step

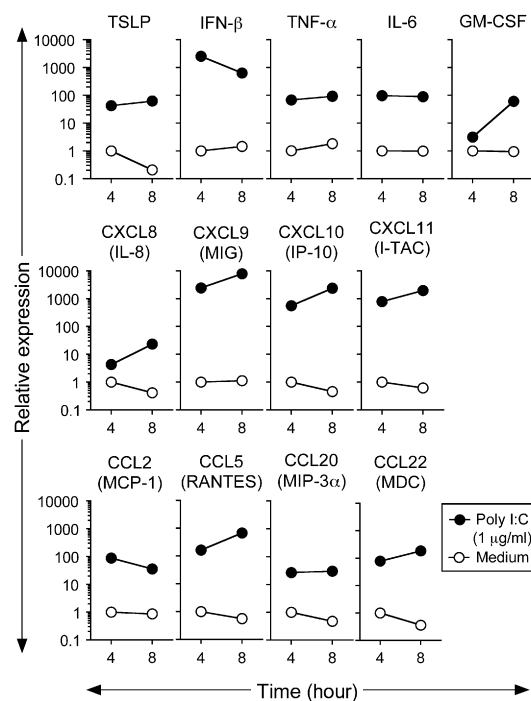


FIG 2. Poly I:C induced upregulation of gene expression of TSLP and other cytokines and chemokines in keratinocytes. Expression of mRNA at 4 and 8 hours after stimulation was analyzed by means of quantitative real-time PCR and is presented as fold change relative to medium at 4 hours. Data shown are representative of 3 independent experiments. MIG, Monokine induced by IFN- γ ; IP-10, IFN-inducible protein 10; I-TAC, IFN-inducible T-cell α chemoattractant; MCP-1, monocyte chemoattractant protein 1; MIP-3 α , macrophage inflammatory protein 3 α ; MDC, macrophage-derived chemokine.

3 we examined whether type I IFNs (IFN- α and IFN- β) promote the dsRNA-induced release of TSLP (Fig 5) because type I IFNs are known to upregulate gene expression of TLR3, a receptor for dsRNA.²⁷ In step 4 we examined whether nonatopic cytokine milieu that could be provided through interaction with T_H1 , Treg, or T_H17 cells regulate the dsRNA-induced release of TSLP (Fig 6). T_H2 cytokines (IL-4 and IL-13), TNF- α , or type I IFNs (IFN- α and IFN- β) enhanced the dsRNA-induced release of TSLP (Figs 3, 4, and 5), whereas IFN- γ , TGF- β , or IL-17 suppressed it (Fig 6). Taken together, the results imply that viral dsRNA and a T_H2 cytokine milieu might promote T_H2 -type inflammation

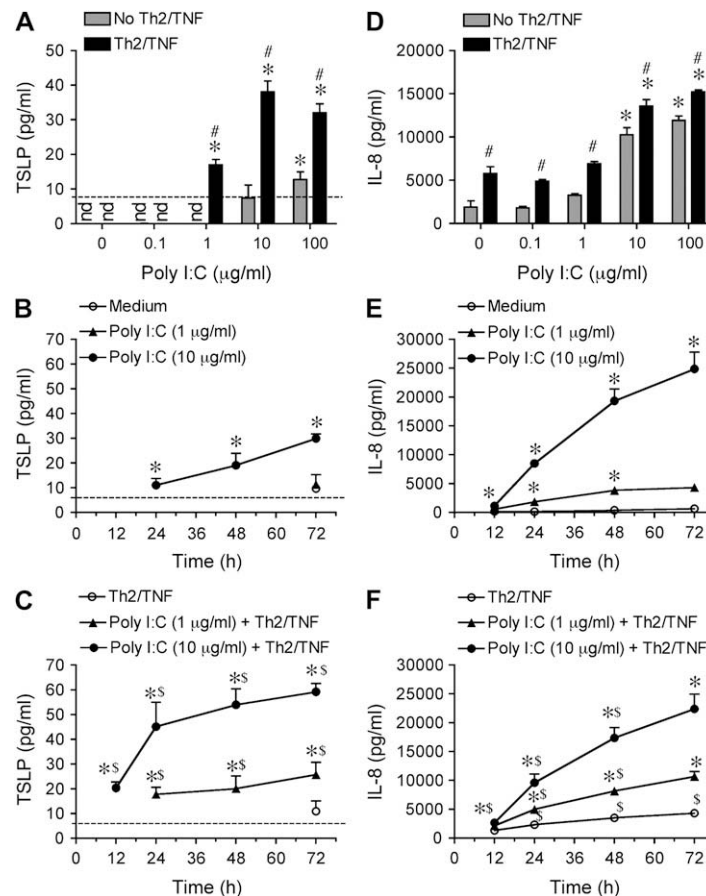


FIG 3. Time course and dose dependency of poly I:C-induced release of TSLP from keratinocytes in the absence or presence of T_H2 /TNF cytokines: **A-C**, TSLP; **D-F**, IL-8. nd, Less than the minimum detection limit (broken line). * $P < .05$ versus without poly I:C by means of ANOVA. # $P < .05$ and \$ $P < .05$ versus without T_H2 /TNF cytokines by means of t test.

through an induction of TSLP expression, suggesting that a vicious cycle exists between AD with T_H2 -type inflammation and viral infections and a possible blockade of this cycle by other T_H1 , Treg, or T_H17 cytokine milieu.

TSLP's release from keratinocytes stimulated with poly I:C (Fig 1) suggests a possibility that viral dsRNA contributes to the T_H2 skewing in sensitization to environmental allergens. Although there have been few clinical investigations of whether exposure to viral components during or before the period of sensitization to allergens contributes to the establishment of T_H2 -skewed immune responses to allergens in patients with AD, a recent longitudinal survey with 9744 children followed up from birth to 3 to 15 years reported an approximately 2-fold increased incidence of AD after measles, mumps, and rubella vaccination and after measles infection compared with that seen in unexposed children.²⁸ Lebre et al²⁹ suggested that factors released from poly I:C-stimulated keratinocytes activate DCs to promote the generation of T_H1 cells on the basis of their observation that DCs, which were induced to mature by LPS in the presence of the culture supernatant of poly I:C-stimulated keratinocytes, caused more T_H1 skewing in the differentiation from naive T cells. However, according to recent progress in the understanding of DC activation,³⁰ whether the culture supernatant of dsRNA-stimulated keratinocytes induces the typical phenotype of TSLP DCs in the conditions without LPS and

whether the DCs induce T_H2 skewing would be addressed in a future study.

Patients with AD, in whom T_H2 -skewed allergic sensitization is already established, are susceptible to viral dissemination,¹⁶ as supported by a study with a mouse model of AD.³¹ Recently, Howell et al¹⁹ demonstrated that a T_H2 cytokine milieu contributes to a reduction in the expression of filaggrin, which is critical for an effective skin barrier¹⁸ and the genetic mutations of which are a major predisposing factor for AD.³² They also reported that the T_H2 cytokine milieu enhances viral replication in the skin of patients with AD through downregulation of the expression of an antiviral peptide, LL-37, which is induced by viral infection or poly I:C in healthy individuals or patients with psoriasis.²⁰ In the present study we demonstrated that T_H2 cytokines, TNF- α , or both upregulate the poly I:C-induced release of TSLP from keratinocytes (Fig 3, A-C, and Fig 4, A-C). Taken together, the T_H2 cytokine milieu in the skin promotes viral infections and replication by reducing the barrier function^{19,31} and expression of the antiviral peptide,²⁰ and the viral replication could exacerbate the T_H2 -type inflammation through viral dsRNA-induced release of TSLP from keratinocytes synergistically with the T_H2 microenvironment, the inflammatory microenvironment, or both. This implies a novel concept: a vicious cycle between AD and viral infection. A T_H2 cytokine milieu, inflammatory cytokine milieu, or both can be provided to keratinocytes through interaction with

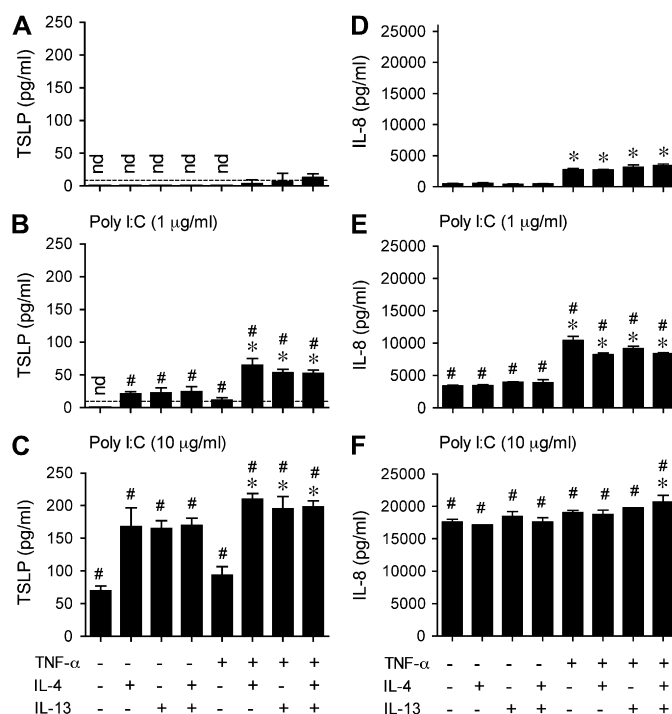


FIG 4. TNF- α and T_H2 cytokines synergistically upregulated poly I:C-induced release of TSLP from keratinocytes: **A-C**, TSLP; **D-F**, IL-8. *nd*, Less than the minimum detection limit (*broken line*). **P* < .05 versus without the T_H2/TNF cytokines by means of ANOVA. #*P* < .05 versus without poly I:C by means of the *t* test.

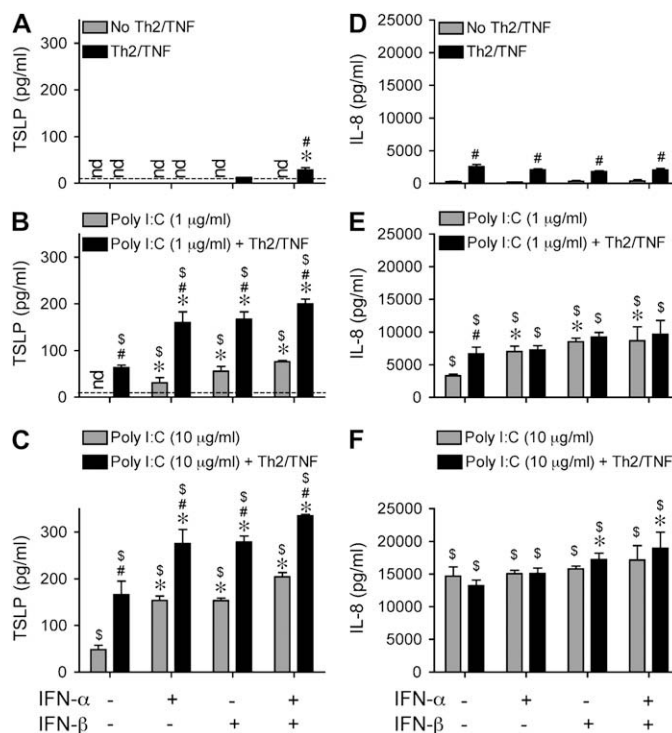


FIG 5. Type I IFNs upregulated poly I:C-induced release of TSLP from keratinocytes: **A-C**, TSLP; **D-F**, IL-8. *nd*, Less than the minimum detection limit (*broken line*). **P* < .05 versus without the type I IFNs by means of ANOVA. #*P* < .05 versus without the T_H2/TNF cytokines and \$*P* < .05 versus without poly I:C by means of the *t* test.

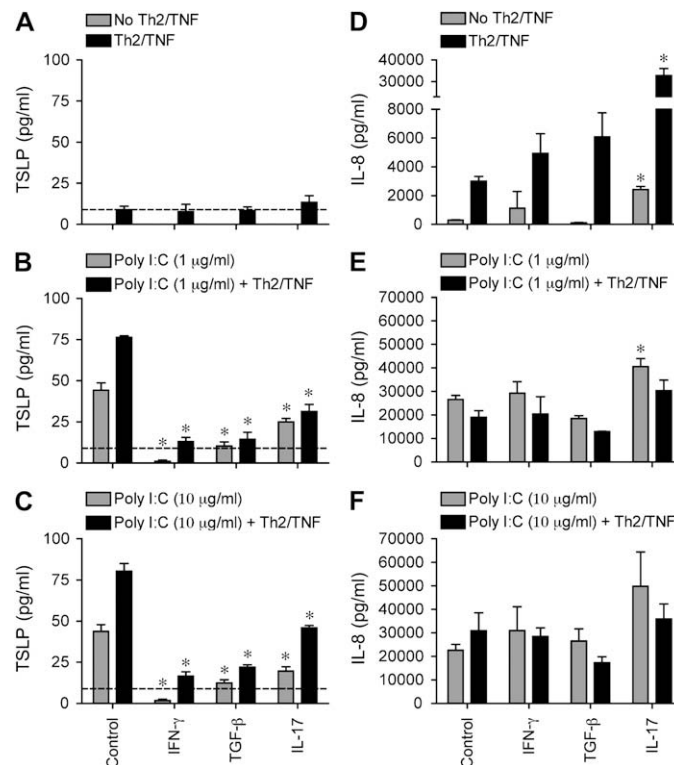


FIG 6. IFN- γ , TGF- β , and IL-17 suppressed poly I:C-induced release of TSLP from keratinocytes: **A-C**, TSLP; **D-F**, IL-8. *nd*, Less than the minimum detection limit (broken line). * $P < .05$ versus the control by means of ANOVA.

allergen-specific T_H2 cells, mast cells, and basophils. The release of TSLP induced by viral dsRNA could be more effective in AD skin than in healthy skin because AD is associated with T_H2 inflammation^{24,25} and allergen-specific IgE, which stimulates mast cells and basophils to produce such cytokines through the high-affinity IgE receptor on exposure to allergens.³³ Promotion of the poly I:C-induced release of TSLP by the type I IFNs (Fig 5, B and C) could be explained by upregulation of the gene expression of TLR3 by the type I IFNs in keratinocytes.²⁷ Type I IFNs can be induced by dsRNA in keratinocytes (Fig 2, IFN- β).^{27,29} This second cycle could also contribute to the enhancement of keratinocyte response to dsRNA to release more TSLP.

We also examined whether the T_H1 , Treg, and T_H17 cytokines enhance or suppress the poly I:C-induced release of TSLP from keratinocytes (Fig 6, B and C). IFN- γ is a T_H1 cytokine that can inhibit T_H2 responses³⁴ and upregulate filaggrin expression in keratinocytes.¹⁹ Clark et al³⁵ reported that in normal skin most of a specialized subtype of T_H cells expressing the skin-homing receptor cutaneous lymphocyte-associated antigen are IFN- γ -expressing T_H1 cells. IL-10 and TGF- β are anti-inflammatory factors mediating the suppressive effect of some subsets of Treg cells.³⁶⁻³⁸ Akdis et al³⁸ reported that IL-10- and TGF- β -expressing T_R1 cells represent the dominant subset for common environmental allergens in healthy individuals, and in contrast, there is a high frequency of allergen-specific T_H2 cells in allergic individuals. IL-17 and IL-22 are representative cytokines produced by recently identified T_H17 cells, which might regulate skin innate immunity.^{39,40} IFN- γ , TGF- β , or IL-17 significantly suppressed the release in the absence and presence of the T_H2 /TNF cytokines (Fig 6, B and C). Our results suggest

that the interaction of keratinocytes with T_H1 , Treg, or T_H17 cells could prohibit TSLP's release from viral dsRNA-stimulated keratinocytes, even in the presence of T_H2 /TNF cytokines.

In primary human airway epithelial cells a recent report has demonstrated that the poly I:C-induced upregulation of *TSLP* mRNA expression is dependent on nuclear factor κ B and interferon regulatory factor 3 signaling through TLR3.¹³ A recent report that TSLP's release from human skin explants, which were obtained from healthy donors and stimulated with T_H2 /TNF cytokines, was not inhibited by IFN- γ , IL-10, and TGF- β ¹⁵ suggests that our finding of an inhibitory effect in primary human keratinocytes by IFN- γ and TGF- β (Fig 6, B and C) could be specific to the dsRNA-induced TSLP release. Mechanisms for the inhibition of the dsRNA-induced release of TSLP by IFN- γ , TGF- β , and IL-17 (Fig 6, B and C) and the dsRNA-independent, IFN- α/β -dependent release of TSLP (Fig 5, A) would be addressed in future studies. Improvements to our system using primary keratinocytes for a more effective induction of TSLP expression by T_H2 /TNF cytokines would be useful for investigations of the molecular mechanisms of TSLP's regulation by the promoting and inhibitory cytokines.

How TSLP expression is triggered in keratinocytes had not been well investigated. In the present study we suggested that exposure to viral dsRNA might induce TSLP's release in the epidermis through the stimulation of keratinocytes. Viral infections can be promoted by a T_H2 cytokine milieu in AD skin,^{19,20} and in turn, the T_H2 cytokine milieu and viral dsRNA could synergistically induce the expression of TSLP in the epidermis. This might be similar in the airway epithelium.¹³ In airway epithelial cells peptidoglycan, a component of gram-positive bacteria, has been also reported to induce TSLP's release, although less

effectively than dsRNA.¹¹ During evaluation of the present article, Harada et al⁴¹ reported that a genetic polymorphism of the *TSLP* gene is functional in enhancement of TSLP promoter activity in poly I:C-stimulated normal human bronchial epithelial cells.

What other stimuli are essential for the induction of TSLP expression in the initial process of sensitization to environmental allergens? Interestingly, cysteine protease activity of highly purified recombinant house dust mite major group 1 allergens, which are the most clinically relevant allergens worldwide, crucially contributes to the sensitization process in mice,^{42,43} and very recently, Sokol et al⁴⁴ have demonstrated that protease activity of papain, an occupational allergen homologous to the mite group 1 allergens, initiates T_H2 sensitization in mice through activation of basophils to express T_H2-inducing cytokines, including IL-4 and TSLP. Thus the activation of keratinocytes, airway epithelial cells, or basophils now has been suggested to contribute to allergic sensitization, exacerbation, and/or perpetuation of allergic diseases through the induction of TSLP expression by environmental triggers. Further investigation of the regulation of TSLP expression in these types of cells by environmental and endogenous factors is needed to elucidate the pathogenesis of allergic diseases and to prevent their development.

In summary, the effect of the TLR3 ligand on primary human keratinocytes in the present study suggests contribution of viral dsRNA to skin inflammations under the influence of a cytokine milieu. We demonstrated that poly I:C stimulated the release of TSLP by keratinocytes (Figs 1 and 2) and that T_H2 cytokines, TNF- α , or type I IFNs enhanced it (Figs 3-5), whereas T_H1, Treg, or T_H17 cytokines suppressed it (Fig 6). These *in vitro* results imply that viral infections and T_H2 and inflammatory cytokine milieus, which could be provided to keratinocytes through interaction with T_H2 cells, mast cells, basophils, and so on, might promote T_H2 inflammation through the expression of TSLP in AD skin and suggest that a vicious cycle exists between AD and viral infections and a possible blockade of this cycle by other cytokine milieus, which could be provided through interaction with T_H1, Treg, or T_H17 cells. Poly I:C upregulated gene expression of various molecules, including not only TSLP, the master switch for T_H2 responses, but also T_H1-attracting chemokines (CXCL9/monokine induced by IFN- γ , CXCL10/IFN-inducible protein 10, and CXCL11/IFN-inducible T-cell α chemoattractant) in keratinocytes *in vitro* (Fig 2). How dsRNA actually affects skin inflammations is an issue to be investigated in future studies.

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Clinical implications: The effect of the TLR3 ligand on keratinocytes suggests contribution of viral dsRNA to skin inflammations under the influence of a cytokine milieu. Exposure to viral dsRNA might cause development or exacerbation of AD through induction of TSLP in keratinocytes, which can be enhanced or blocked by interaction with cytokine-producing cells.

REFERENCES

- Liu YJ. Thymic stromal lymphopoietin and OX40 ligand pathway in the initiation of dendritic cell-mediated allergic inflammation. *J Allergy Clin Immunol* 2007; 120:238-44.
- Liu YJ. Thymic stromal lymphopoietin: master switch for allergic inflammation. *J Exp Med* 2006;203:269-73.
- Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002;3:673-80.
- Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, et al. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J Immunol* 2005;174:8183-90.
- Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med* 2005;202:1213-23.
- Ebner S, Nguyen VA, Forstner M, Wang YH, Wolfram D, Liu YJ, et al. Thymic stromal lymphopoietin converts human epidermal Langerhans cells into antigen-presenting cells that induce proallergic T cells. *J Allergy Clin Immunol* 2007; 119:982-90.
- Wang YH, Ito T, Wang YH, Homey B, Watanabe N, Martin R, et al. Maintenance and polarization of human TH2 central memory T cells by thymic stromal lymphopoietin-activated dendritic cells. *Immunity* 2006;24:827-38.
- Yoo J, Omori M, Gyarmati D, Zhou B, Aye T, Brewer A, et al. Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. *J Exp Med* 2005;202:541-9.
- Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB, et al. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat Immunol* 2005;6:1047-53.
- Al-Shami A, Spolski R, Kelly J, Keane-Myers A, Leonard WJ. A role for TSLP in the development of inflammation in an asthma model. *J Exp Med* 2005;202: 829-39.
- Allakhverdi Z, Comeau MR, Jessup HK, Yoon BR, Brewer A, Chartier S, et al. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. *J Exp Med* 2007;204:253-8.
- Rochman I, Watanabe N, Arima K, Liu YJ, Leonard WJ. Cutting edge: direct action of thymic stromal lymphopoietin on activated human CD4⁺ T cells. *J Immunol* 2007;178:6720-4.
- Kato A, Favoreto S Jr, Avila PC, Schleimer RP. TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells. *J Immunol* 2007;179:1080-7.
- Xu W, He B, Chiu A, Chadburn A, Shan M, Buldys M, et al. Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. *Nat Immunol* 2007;8:294-303.
- Bogiatzi SI, Fernandez I, Bichet JC, Marloie-Provost MA, Volpe E, Sastre X, et al. Cutting edge: proinflammatory and Th2 cytokines synergize to induce thymic stromal lymphopoietin production by human skin keratinocytes. *J Immunol* 2007;178: 3373-7.
- Wollenberg A, Wetzel S, Burgdorf WH, Haas J. Viral infections in atopic dermatitis: pathogenic aspects and clinical management. *J Allergy Clin Immunol* 2003; 112:667-74.
- Ogawa H, Yoshiike T. A speculative view of atopic dermatitis: barrier dysfunction in pathogenesis. *J Dermatol Sci* 1993;5:197-204.
- Segre JA. Epidermal barrier formation and recovery in skin disorders. *J Clin Invest* 2006;116:1150-8.
- Howell MD, Kim BE, Gao P, Grant AV, Boguniewicz M, De Benedetto A, et al. Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol* 2007;120:150-5.
- Howell MD, Gallo RL, Boguniewicz M, Jones JF, Wong C, Streib JE, et al. Cytokine milieu of atopic dermatitis skin subverts the innate immune response to vaccinia virus. *Immunity* 2006;24:341-8.
- Howell MD, Jones JF, Kisich KO, Streib JE, Gallo RL, Leung DY. Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum. *J Immunol* 2004; 172:1763-7.
- Kollisch G, Kalali BN, Voelcker V, Wallich R, Behrendt H, Ring J, et al. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. *Immunology* 2005; 114:531-41.
- Lebre MC, van der Aar AM, van Baarsen L, van Capel TM, Schuitemaker JH, Kapsenberg ML, et al. Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. *J Invest Dermatol* 2007;127:331-41.
- Hamid Q, Boguniewicz M, Leung DY. Differential in situ cytokine gene expression in acute versus chronic atopic dermatitis. *J Clin Invest* 1994;94:870-6.
- Teraki Y, Hotta T, Shiohara T. Increased circulating skin-homing cutaneous lymphocyte-associated antigen (CLA)⁺ type 2 cytokine-producing cells, and decreased CLA⁺ type 1 cytokine-producing cells in atopic dermatitis. *Br J Dermatol* 2000;143:373-8.

26. Nelms K, Keegan AD, Zamorano J, Ryan JJ, Paul WE. The IL-4 receptor: signaling mechanisms and biologic functions. *Annu Rev Immunol* 1999;17:701-38.
27. Tohyama M, Dai X, Sayama K, Yamasaki K, Shirakata Y, Hanakawa Y, et al. dsRNA-mediated innate immunity of epidermal keratinocytes. *Biochem Biophys Res Commun* 2005;335:505-11.
28. Olesen AB, Juul S, Thestrup-Pedersen K. Atopic dermatitis is increased following vaccination for measles, mumps and rubella or measles infection. *Acta Derm Venereol* 2003;83:445-50.
29. Lebre MC, Antons JC, Kalinski P, Schuitemaker JH, van Capel TM, Kapsenberg ML, et al. Double-stranded RNA-exposed human keratinocytes promote Th1 responses by inducing a Type-1 polarized phenotype in dendritic cells: role of keratinocyte-derived tumor necrosis factor alpha, type I interferons, and interleukin-18. *J Invest Dermatol* 2003;120:990-7.
30. MacDonald AS, Maizels RM. Alarming dendritic cells for Th2 induction. *J Exp Med* 2008;205:13-7.
31. Scott JE, ElKhal A, Freyschmidt EJ, MacArthur DH, McDonald D, Howell MD, et al. Impaired immune response to vaccinia virus inoculated at the site of cutaneous allergic inflammation. *J Allergy Clin Immunol* 2007;120:1382-8.
32. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 2006;38:441-6.
33. Kawakami T, Galli SJ. Regulation of mast-cell and basophil function and survival by IgE. *Nat Rev Immunol* 2002;2:773-86.
34. O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 1998;8:275-83.
35. Clark RA, Chong B, Mirchandani N, Brinster NK, Yamanaka K, Dowgiert RK, et al. The vast majority of CLA+ T cells are resident in normal skin. *J Immunol* 2006;176:4431-9.
36. Taylor A, Verhagen J, Blaser K, Akdis M, Akdis CA. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. *Immunology* 2006;117:433-42.
37. Akdis M, Blaser K, Akdis CA. T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. *J Allergy Clin Immunol* 2005;116:961-8.
38. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Cramer R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004;199:1567-75.
39. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 2006;203:2271-9.
40. Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 2007;8:950-7.
41. Harada M, Hirota T, Jodo AI, Doi S, Kameda M, Fujita K, et al. Functional analysis of the thymic stromal lymphopoietin variants in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2008 [Epub ahead of print].
42. Kikuchi Y, Takai T, Kuhara T, Ota M, Kato T, Hatanaka H, et al. Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p1 to sensitization toward IgE and IgG responses. *J Immunol* 2006;177:1609-17.
43. Kikuchi Y, Takai T, Ota M, Kato T, Takeda K, Mitsuishi K, et al. Application of immunoreaction enhancer solutions to an enzyme-linked immunosorbent assay for antigen-specific IgE in mice immunized with recombinant major mite allergens or ovalbumin. *Int Arch Allergy Immunol* 2006;141:322-30.
44. Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat Immunol* 2008;9:310-8.