Interleukin 33 Is Induced by Tumor Necrosis Factor α and Interferon γ in Keratinocytes and Contributes to Allergic Contact Dermatitis

K Taniguchi,1,2 S Yamamoto,1 E Hitomi,1 Y Inada,1 Y Suyama,1 T Sugioka,2 Y Hamasaki1

1Department of Pediatrics, Faculty of Medicine, Saga University, Saga, Japan
2Community Medical Support Institute, Faculty of Medicine, Saga University, Saga, Japan

Abstract

Background: Interleukin (IL) 33, a novel member of the IL-1 family, is produced mainly by epithelial cells and endothelial cells in response to various types of stress, including necrosis. The effects of IL-33 on the immune cells involved in allergic contact dermatitis have recently been revealed in vitro. However, in vivo, the induction mechanism and function of IL-33 are not fully understood.

Objectives: Our objectives were to investigate induction of IL-33 in keratinocytes and to evaluate the functions of IL-33 and its inducers in a murine model of allergic contact dermatitis.

Material and Methods: KERTr cells, a human keratinocyte cell line, were cultured with various cytokines, including tumor necrosis factor (TNF) α and interferon (IFN) γ. IL-33 expression was detected using quantitative reverse transcriptase polymerase chain reaction, immunocytochemistry, and Western blotting. The functions of IL-33, TNF-α, and IFN-γ in allergic contact dermatitis were evaluated using a murine model.

Results: TNF-α and IFN-γ induced expression of IL-33 mRNA and protein in KERTr cells. Blockade of IL-33 attenuated swelling in the ears of the experimental mice. Similar effects were noted for blockade of TNF-α and IFN-γ in these mice.

Conclusions: TNF-α and IFN-γ induce expression of IL-33, and IL-33produced by keratinocytes contributes to allergic contact dermatitis. Blockade of IL-33, TNF-α, and IFN-γ could represent novel and potent strategies to treat allergic contact dermatitis.

Key words: Interleukin 33. Keratinocyte. Tumor necrosis factor. Interferon. Allergic contact dermatitis.

Resumen

Antecedentes: La Interleucina 33 (IL-33), un nuevo miembro de la familia de la IL-1, es producida fundamentalmente por las células epiteliales y endoteliales en respuesta a diferentes estímulos, incluyendo la necrosis. Recientemente se han confirmado los efectos de esta IL sobre las células del sistema inmunológico in vitro en pacientes con dermatitis de contacto, aunque los mecanismo y función in vivo de la IL-33 no son bien conocidos.

Objetivos: El objetivo de este estudio fue analizar los factores que podrían inducir IL-33 en queratinocitos y evaluar las funciones de esta citocina y de sus inductores en un modelo murino de dermatitis alérgica de contacto.

Métodos: Para ello se cultivaron células KERTr, una línea celular de queratinocitos humanos, en presencia de varias citocinas, incluyendo TNF-α e IFN-γ. La expresión de IL-13 se detectó mediante PCR cuantitativa a tiempo real, inmunocitoquímica e inmunobloting. Así mismo se evaluó la función de IL-33, TNF-α, e IFN-γ en el modelo murino.

Resultados: En cuanto a los resultados obtenidos TNF-α e IFN-γ indujeron la expresión de mRNA y expresión de proteína en las células KERTr. El bloqueo de IL-33 atenua la inflamación en la dermatitis de contacto murina.

Conclusiones: En conclusión, TNF-α y IFN-γ son inductores de la producción de IL-33, y además esta citocina producida por los queratinocitos contribuye a la expresión de dermatitis alérgica de contacto. El bloqueo de no solo IL-33, sino también de TNF-α y IFN-γ podría representar una modalidad terapéutica nueva y potente en la dermatitis alérgica de contacto.

Introduction

Interleukin (IL) 33 is a cytokine of the IL-1 family IL-1F11. It acts as a specific extracellular ligand for the orphan IL-1 receptor–related protein ST2 [1]. Epithelial cells and endothelial cells were reported to be the main cellular sources of IL-33 [1-3]. IL-33 is released by cells undergoing necrosis and, in this respect, is thought to function as a damage-associated molecular pattern and alarmin [3,4]. ST2, the receptor for IL-33, is expressed on various immune cells, including type 2 helper T cells (TH2) [5], CD8+ T cells [6], mast cells [7], eosinophils [8], basophils [9], natural killer (NK) cells, NKT cells [10], and dendritic cells [11]. IL-33 induces production of TH2 cytokines (including IL-5 and IL-13) from TH2 cells, eosinophils, basophils, NK cells, and NKT cells [12]. Dendritic cells stimulated by IL-33 promote differentiation of TH2 cells [13]. Natural killer helper cells [14] and nuocytes [15] have recently attracted attention because they produce abundant TH2 cytokines when stimulated by IL-33. Therefore, IL-33 is closely involved in TH2 immune responses. In fact, IL-33 is associated with a range of allergic diseases, including bronchial asthma [16], allergic rhinitis [17], allergic conjunctivitis [18], anaphylaxis [19], atopic dermatitis [20], and allergic contact dermatitis [21].

Allergic contact dermatitis is a clinical form of contact hypersensitivity. It involves a delayed-type hypersensitivity reaction and is the manifestation of an allergic response caused by contact with allergens [22]. Contact allergens are essentially soluble haptons with physical and chemical properties that allow them to cross the stratum corneum of the skin [22]. The mechanisms by which these reactions occur are complex. Allergic contact dermatitis involves a cellular immune response mediated by IL-4- and IL-13-producing TH2 cells and by interferon (IFN)–γ–producing TH1 cells [23]. Tumor necrosis factor (TNF) α, which is produced mainly by monocytes and macrophages, is also involved in the pathogenesis of allergic contact dermatitis [22]. Thus, TH1 and TH2 immune responses coexist in allergic contact dermatitis [22,23].

IL-33 plays a key role in the pathogenesis of allergic contact dermatitis by promoting TH2 immune responses [21]. However, the induction mechanism and function of IL-33 in allergic contact dermatitis are not fully understood. In the present study, we investigated induction of IL-33 in keratinocytes and evaluated the functions of IL-33 and its inducers in a murine model of allergic contact dermatitis.

Material and Methods

Cell Culture

KERTr cells, which are a human keratinocyte cell line, were purchased from the American Type Culture Collection (ATCC). The cells were cultured in keratinocyte serum-free medium supplemented with 0.05 mg/mL of bovine pituitary extract and 35 ng/mL of human recombinant epidermal growth factor (all from Invitrogen).

Quantitative Reverse Transcriptase Polymerase Chain Reaction

KERTr cells were seeded at 3×10⁴ cells/well on 6-well plates (Thermo Fisher Scientific) and incubated overnight. The cells were then incubated with recombinant TNF-α, IFN-γ, IL-6, granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-1α, IL-1β, IL-4, IL-13, IL-17A (PeproTech), and polyinosinic-polycytidylic acid potassium salt (poly I:C) (Sigma-Aldrich). RNA was isolated from the KERTr cells using TRIzol (Invitrogen) and reverse-transcribed with ReverTra Ace (Toyobo) and deoxynucleotide triphosphate (Promega) according to the manufacturer’s protocol. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo) and an Applied Biosystems StepOnePlus Real-Time PCR System (Life Technologies Japan). Oligonucleotide primers were designed using Primer BLAST software (National Center for Bioinformatics).

Western Blotting

KERTr cells were incubated with 200 ng/mL recombinant TNF-α or 20 ng/mL recombinant IFN-γ for various periods. The cells were then lysed using radioimmunoprecipitation assay buffer. Western blotting was carried out with a goat polyclonal anti–IL-33 primary antibody (R&D Systems), followed by a hors eradish peroxidase (HRP)–conjugated rabbit anti-goat IgG secondary antibody (Santa Cruz Biotechnology). As an internal control, Western blotting was carried out with a mouse monoclonal antialgyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibody (Santa Cruz Biotechnology), followed by an HRP-conjugated goat antimouse IgG secondary antibody (Santa Cruz Biotechnology). The membranes were developed with an ECL Plus Western Blotting Detection System (GE Healthcare).

Immunocytochemistry

KERTr cells were seeded at 1×10⁴ cells/well on 8-well Lab-Tek Chamber Slides (Thermo Fisher Scientific) and incubated overnight. One set of cells was then incubated with and without 200 ng/mL recombinant TNF-α for 6 hours, while another set of cells was incubated with and without 20 ng/mL recombinant IFN-γ for 12 hours. All the cultured cells were fixed and stained with a mouse monoclonal anti–IL-33 primary antibody (Enzo Life Sciences), followed by an Alexa Fluor 488-conjugated goat antimouse IgG secondary antibody (Invitrogen). Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (Dojindo). An IgG1 isotype control antibody (Enzo Life Sciences) was also evaluated.

Sensitization and Induction of Allergic Contact Dermatitis

Animal experiments were undertaken following the guidelines for the care and use of experimental animals of the Japanese Association for Laboratory Animal Science (1987). Six-week-old female C57BL/6 mice were purchased from Kyudo. The protocol for preparing the murine model of allergic contact dermatitis was reported previously [24]. Briefly, mice
were painted with 100 μL of 5% 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxa) in ethanol on their shaved abdomen on day 0. Oxa challenges were performed on days 5, 7, 9, 11, and 13 after sensitization. The right ears of the mice were painted with 0.1% oxa in ethanol, while the left ears were painted with ethanol alone. An antimouse IL-33 antibody (functional grade purified; Medical & Biological Laboratory), antimouse TNF-α antibody (functional grade purified; eBiosciences), or antimouse IFN-γ antibody (functional grade purified; eBiosciences) was subcutaneously injected into the ears on day 9. An IgG1 isotype control antibody (Abcam) was also evaluated. The mice were categorized as follows: group 1, not sensitized, oxa-challenged, no antibody injected; group 2, oxa-sensitized, oxa-challenged, no antibody injected; group 3, oxa-sensitized, oxa-challenged, isotype control IgG1 injected; group 4, oxa-sensitized, oxa-challenged, anti–IL-33 antibody injected; group 5, oxa-sensitized, oxa-challenged, anti–TNF-α antibody injected; group 6, oxa-sensitized, oxa-challenged, anti–IFN-γ antibody injected.

**Measurement of Serum IgE**

Mice were euthanized by intraperitoneal injection of Isozol (Nichiiko) on day 15 after sensitization. Blood samples were collected. The serum IgE concentrations were measured with a Mouse IgE ELISA Kit (Shibayagi) according to the manufacturer’s protocol.

**Measurement of Ear Thickness**

Ear thickness was measured using a Peacock dial thickness gauge G-1A (Ozaki). Ear swelling was calculated as follows: [thickness of ears after oxa applications – thickness of ears before oxa applications] – [thickness of ears after vehicle applications – thickness of ears before vehicle applications].

**Histology and Immunohistochemistry**

Ear tissues were fixed and immunostained. Expression of IL-33 protein was investigated by means of cross-sections stained with a mouse monoclonal anti–IL-33 antibody [Nessy-1] (Biotin) (Abcam) and evaluated using a Histofine SAB-PO (M) Kit and a Histofine DAB Substrate Kit (Nichirei, Tokyo, Japan) according to the manufacturer’s protocols. Nuclei were stained with hematoxylin.

**Statistical Analysis**

Statistical analyses were performed using Prism 5.0 (GraphPad Software). Data are presented as mean (SD). The significance of differences between values was assessed using an unpaired t test. P values of <.05 were considered statistically significant.

**Results**

**TNF-α and IFN-γ Induce Expression of IL-33 mRNA in KERTr Cells**

In order to investigate which cytokines could induce expression of IL-33 mRNA, we stimulated KERTr cells using various cytokines associated with allergic contact dermatitis. qRT-PCR revealed that TNF-α and IFN-γ were able to induce expression of IL-33 mRNA (Figure 1). The expression of IL-33 mRNA induced by TNF-α peaked at 200 ng/mL (Figure 1A). In the kinetics analysis, expression of IL-33 mRNA induced by TNF-α peaked at 3 hours after stimulation (Figure 1B); expression of IL-33 mRNA induced by IFN-γ peaked at 20 ng/mL (Figure 1C). In the kinetics analysis, expression of IL-33 mRNA induced by IFN-γ peaked at 6 hours after stimulation (Figure 1D). These findings showed that TNF-α and IFN-γ induced IL-33 mRNA expression in KERTr cells. Other cytokines, including IL-6, GM-CSF, IL-1α, IL-1β, IL-4, IL-13, and IL-17, and poly (I:C), a mimic of double-stranded RNA, did not induce IL-33 mRNA expression (data not shown).
IL-33 in Allergic Contact Dermatitis


Figure 2. TNF-α and IFN-γ induce expression of IL-33 protein. A, KERTr cells were incubated with 200 ng/mL TNF-α or phosphate-buffered saline (PBS) for 6 hours. B, KERTr cells were incubated with 20 ng/mL IFN-γ or PBS for 12 hours. Scale bars, 10 μm. C, KERTr cells were incubated with 200 ng/mL TNF-α or 20 ng/mL IFN-γ for the indicated times. TNF indicates tumor necrosis factor; IFN, interferon; IL, interleukin; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

TNF-α and IFN-γ Induce Expression of IL-33 Protein in KERTr Cells

We performed immunocytochemistry and Western blotting analysis to investigate whether TNF-α and IFN-γ could induce IL-33 protein expression. In KERTr cells incubated without these cytokines, IL-33 protein was located in the nucleus (Figure 2A and B). In KERTr cells incubated with TNF-α, expression of IL-33 protein was increased, and its location shifted from the nucleus to the cytoplasm (Figure 2A). Similarly, in KERTr cells incubated with IFN-γ, expression of IL-33 protein was increased, and the protein showed the same translocation pattern as that of stimulation with TNF-α (Figure 2B). These findings suggested that IL-33 was constitutively expressed in the nucleus and shifted to the cytoplasm when its expression was upregulated.

We further examined expression of IL-33 protein using Western blotting. In KERTr cells incubated with TNF-α, upregulation of IL-33 protein began 3 hours after stimulation, peaked at 6 hours, and then gradually disappeared (Figure 2C). In KERTr cells incubated with IFN-γ, upregulation of the IL-33 protein began at 6 hours after stimulation and then gradually disappeared (Figure 2C). We investigated whether IL-33 was secreted into the culture supernatants. However, we did not detect any IL-33 in the supernatants by enzyme-linked immunosorbent assay (ELISA) (data not shown).

Preparation of the Murine Model of Allergic Contact Dermatitis

We used a murine model to evaluate the functions of IL-33, TNF-α, and IFN-γ in allergic contact dermatitis. We confirmed that sensitization was successful by measuring serum IgE concentrations using ELISA. The serum IgE levels obtained from oxa-sensitized mice (groups 2 to 6) were significantly higher than those obtained from nonsensitized mice (group 1) (Figure 3), suggesting that the model was successful. Ear swelling in group 2 mice was significantly greater than in group 1 mice (Figure 4). Immunohistochemistry revealed that expression of IL-33 protein was upregulated in group 2 mice compared with group 1 mice (Figure 5).

Blockade of IL-33 Attenuates Ear Swelling

In order to evaluate the function of IL-33, we administered anti–IL-33 antibody to the mice in our model. Ear swelling was increased in group 3 mice (isotype control IgG1–injected), as well as in group 2 mice. However, in the anti–IL-33 antibody–injected mice (group 4), ear swelling was not increased and was significantly milder than in group 3 mice (Figure 4). Immunohistochemistry showed that expression of IL-33 protein in keratinocytes was upregulated...
in group 3 mice compared with mice from group 1, as well as group 2 (Figure 5). Furthermore, expression of IL-33 protein in keratinocytes was not upregulated in group 4 mice (Figure 5). These findings demonstrated that blockade of IL-33 attenuated ear swelling. Incidentally, the serum IgE level in group 4 mice showed no significant differences compared with group 3 mice (Figure 3), suggesting that blockade of IL-33 could not attenuate oxa sensitization.

**Blockade of TNF-α and IFN-γ Attenuates Ear Swelling**

We administered an anti–TNF-α antibody or anti–IFN-γ antibody to the mice in our model in order to evaluate the functions of TNF-α and IFN-γ. In the anti–TNF-α antibody–injected mice (group 5), ear swelling was not increased and was significantly milder than in group 3 mice (Figure 4). In the anti–IFN-γ antibody–injected mice (group 6), ear swelling was not increased and was significantly milder than in group 3 mice (Figure 4). Immunohistochemistry showed that expression of IL-33 protein in keratinocytes in group 5 and group 6 mice was downregulated to the level of group 4 mice (Figure 5). These findings demonstrated that blockade of TNF-α and IFN-γ attenuated ear swelling in our model. This attenuation could be mediated by downregulation of IL-33 expression. Incidentally, the serum IgE levels obtained from group 5 and group 6 mice showed no significant differences compared with group 3 mice (Figure 3), suggesting that blockade of TNF-α and IFN-γ was unable to attenuate oxa sensitization.

**Discussion**

The contribution of IL-33 to allergic diseases has been widely reported [16-21]. Although the effects of IL-33 on the immune cells that appear in allergic diseases are well known [5-11], the induction mechanism of IL-33 is not fully understood. Very recently, TNF-α and IFN-γ were shown to induce IL-33 expression in normal human epidermal keratinocytes in vitro [25]. Our data support this finding. Consequently, IL-33 plays a role in inflammatory skin diseases involving high levels of TNF-α and IFN-γ. In the present study, IL-33 was constitutively expressed in the nucleus of KERTr cells, where it was upregulated by TNF-α and IFN-γ before entering the cytoplasm. IL-33 is also a secreted protein, and this shift may be part of the process of secretion. However, IL-33 protein was not detected in the cell culture supernatants. Failure to detect secreted endogenous
IL-33 in different types of healthy cells has been reported in various studies [25-27]; secreted IL-33 was only detected once the cells had begun to undergo necrosis.

Our findings provide a mechanism that could explain upregulation of IL-33 in keratinocytes in allergic contact dermatitis. Immune responses can be roughly classified as type 1 and type 2. IFN-γ, which is mainly produced by Th1 cells, and TNF-α, which is mainly produced by monocytes and macrophages, play an active part in type 1 immune responses. On the other hand, IL-4, IL-5, and IL-13, which are produced by Th2 cells, play an active role in type 2 immune responses. IFN-γ has been thought to suppress type 2 immune responses through inhibition of Th2 cell proliferation [28]. However, in chronic inflammation, expression of Th2 cytokines occurs even in type 1 immune responses [29]. We previously reported that IFN-γ had opposite effects on IL-4-induced production of CCL26, a potent chemoattractant for eosinophils, which are effector cells in type 2 immune responses in normal human epidermal keratinocytes [30]. Our data support the coexistence of the properties of type 1 and type 2 immune responses. In the present study, we found that IFN-γ and TNF-α, which play a key role in type 1 immune responses, induced expression of IL-33, which could promote type 2 immune responses in keratinocytes. Our findings provide evidence for both upregulation of IL-33 in keratinocytes and coexistence of type 1 and type 2 immune responses in allergic contact dermatitis.

IL-33 and its receptor ST2 appear to contribute to allergic contact dermatitis. For example, ST2 was upregulated in skin lesions in a contact dermatitis rat model using a representative hapten, 2,4-dinitrofluorobenzene [31]. In an oxazolone-induced murine model of allergic contact dermatitis, inflammation was attenuated in ST2-deficient mice compared with wild-type mice, because activation of B-1 cells via IL-33 and ST2 interactions could not occur [21]. We generated our model under conditions by which a difference in epidermal thickness was observed between the control and experimental groups and edema and hypertrophy of the dermis were not observed. We found that blockade of IL-33 attenuated ear swelling in a murine model of allergic contact dermatitis. Thus, IL-33 is functionally involved in the pathogenesis of allergic contact dermatitis, especially in keratinocytes, the main structural cells in the epidermis. However, blockade of IL-33 did not attenuate sensitization with oxa. Therefore, we suggest that IL-33 is not involved in the sensitization phase, at least in our experiments. IL-33 is expressed in the nucleus of keratinocytes at low levels in the resting phase. Once inflammation occurs, IL-33 can be initially upregulated in keratinocytes and secreted as alarmin, before exerting its effects on immune cells that mediate allergic responses. Thus, inflammation may be facilitated and prolonged in allergic contact dermatitis. Expression levels of TNF-α and IFN-γ are upregulated in allergic contact dermatitis [32]. TNF-α and IFN-γ can promote expression of IL-33 in keratinocytes, thus sustaining chronic inflammation. We further showed that blockade of TNF-α and IFN-γ attenuated allergic contact dermatitis. In addition, expression of IL-33 in keratinocytes was decreased by blockade of TNF-α and IFN-γ. We suggest that blockade of TNF-α and IFN-γ suppresses upregulation of IL-33 expression in keratinocytes, resulting in the attenuation of allergic contact dermatitis. Therefore, both blockade of IL-33 and blockade of TNF-α and IFN-γ could be effective treatments for allergic contact dermatitis.

The main treatments for allergic contact dermatitis are topical and systemic (oral and injected) corticosteroids and antihistamines. These treatments successfully inhibit allergic inflammation, including the infiltration of inflammatory cells into the skin and secretion of various inflammatory cytokines [33]. However, corticosteroids and antihistamines are used to treat symptoms, rather than provide a cure, and are thus unable to completely prevent the inflammation from becoming chronic. The results of the present study suggest that blockade of IL-33, TNF-α, and IFN-γ could become a potent strategy for allergic contact dermatitis even in the chronic phase, in which type 1 and type 2 immune responses coexist.

In conclusion, TNF-α and IFN-γ were able to induce expression of IL-33 in KERTr cells. Blockade of IL-33 and blockade of TNF-α and IFN-γ attenuated allergic contact dermatitis in a murine model. These findings could pave the way for successful treatment of allergic contact dermatitis.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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K Kazuto Taniguchi

Department of Pediatrics
Faculty of Medicine
Saga University
5-1-1 Nabeshima
Saga 849-8501, Japan