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Letter to the Editor

dsRNA induces IL-33 promoter activity through TLR3-EGFR-IRF3 pathway in normal human epidermal keratinocytes

To the Editor

IL-33 is a member of the IL-1 cytokine family and constitutively expressed in endothelial cells and epithelial cells exposed to stimuli. IL-33 was identified as a ligand of the IL-1 receptor family member ST2L. IL-33 activates group2 innate immune cells, mast cells, eosinophils, basophils, NKT cells, dendritic cells, and neutrophils [1]. IL-33 can enhance the Th2 immune reaction to promote cytokines, and it also functions as a nuclear factor to suppress or enhance NF-κB function [2]. Recent studies showed that IL-33 is

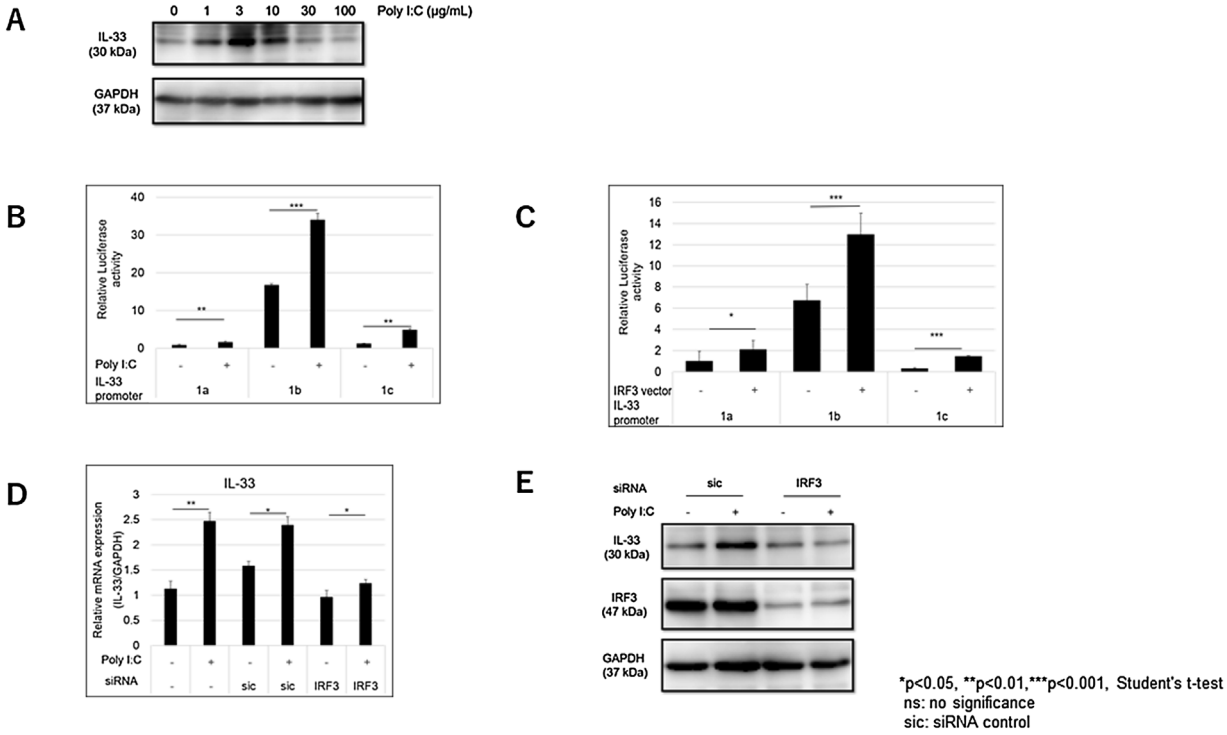

Fig. 1. IL-33 promoter was activated by Poly I:C stimulation and co-transfection of IRF3 in NHEKs.
(A) IL-33 protein expression was analyzed by western blotting. NHEKs were harvested 8 h after treatment with Poly I:C at 1, 3, 10, 300, or 100 μg/mL. GAPDH was used as a loading control.
(B) Promoter activity of IL-33 was induced by Poly I:C. Cells were transfected with 1 μg of each firefly luciferase construct with 50 ng of HSV-thymidine kinase promoter (TK)-driven Renilla luciferase construct incubated for 5 h at 37 °C, and then stimulated with Poly I:C (10 μg/mL). 8 h after stimulation, the cells were collected. Firefly and the Renilla luminescence activity were measured with Glo-Max (Promega). The promoter activity was calculated by dividing firefly luminescence activity by Renilla luminescence activity.
(C) Forced expression of IRF3 induced promoter activity of IL-33. Cells were transfected with firefly luciferase construct of IL-33 promoter, IRF3 expression vector, pBluescript SK, and HSV-thymidine kinase promoter (TK)-driven Renilla luciferase construct. Cells were incubated for 24 h at 37 °C and harvested. Promoter activity was calculated by dividing firefly luminescence activity by Renilla luminescence activity.
(D) siRNA for IRF3 or control oligos were transfected into NHEKs, after which the cells were incubated 72 h to confluence and stimulated with Poly I:C. Cells were harvested 8 h after stimulation. RT-PCR was used to evaluate IL-33 mRNA expression.
(E) IRF3 and IL-33 protein expression was analyzed by western blotting. GAPDH was used as a loading control. Sic: control siRNA. Student *t*-test was applied for statistical evaluation.

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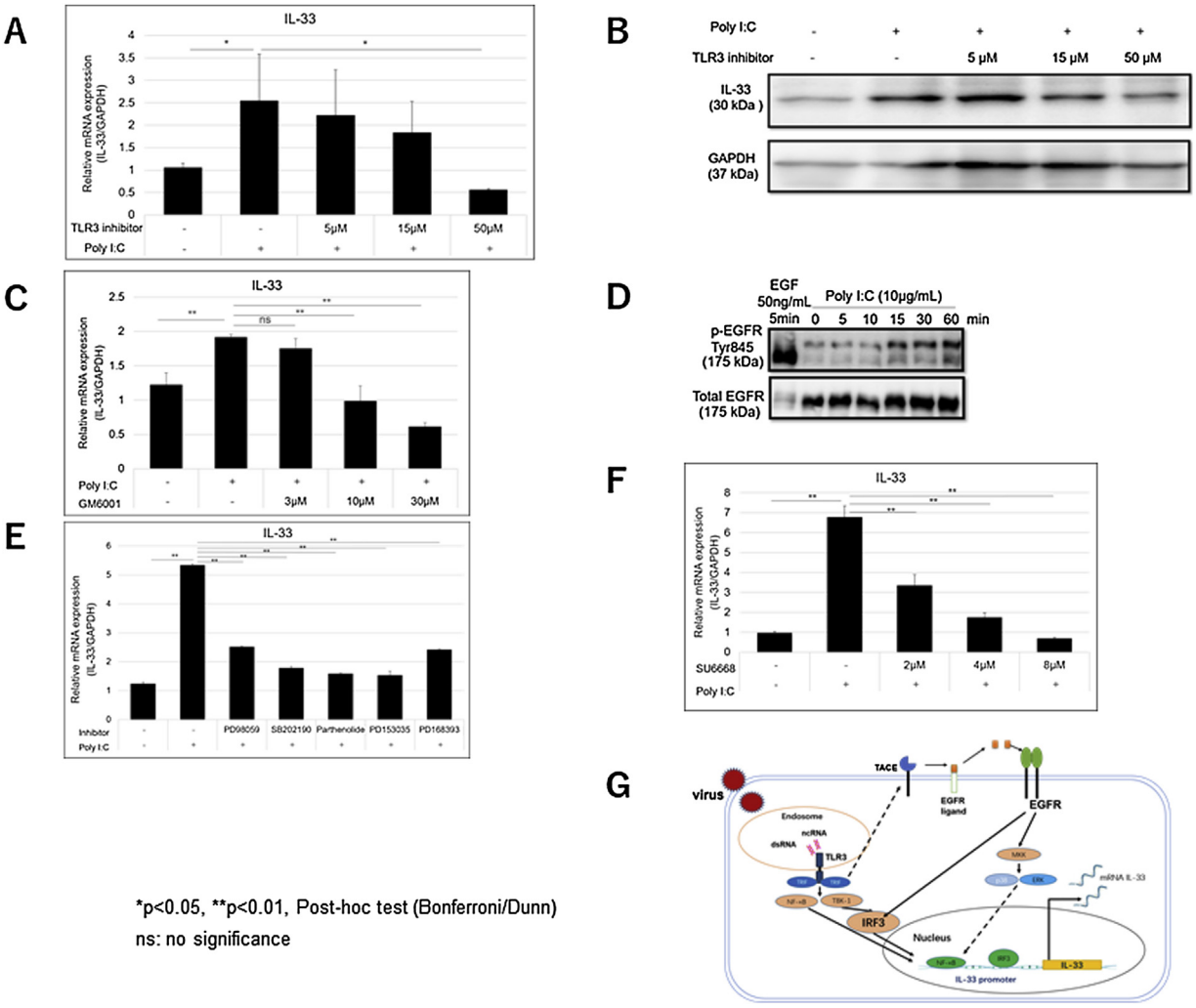

*p<0.05, **p<0.01, Post-hoc test (Bonferroni/Dunn)
ns: no significance

Fig. 2. Several signaling pathways involved in IL-33 induction by TLR3, especially TLR3-EGFR-IRF3 in NHEKs.
(A) IL-33 induction by Poly I:C was suppressed by TLR3 inhibitor by RT-PCR. TLR3 inhibitor was added 30 min before Poly I:C stimulation, and cells were harvested after 8 h of incubation. NHEKs were stimulated with 10 μg/mL of Poly I:C.
(B) IL-33 protein expression was analyzed by western blotting. TLR3 inhibitor was added at the indicated concentration, followed by stimulation with Poly I:C (10 μg/mL). GAPDH was used as a loading control.
(C) IL-33 induction by Poly I:C was suppressed by TACE inhibitor. NHEKs were pre-incubated with GM6001 (TACE inhibitor) for 30 min before stimulation with Poly I:C (10 μg/mL) for 8 h. IL-33 mRNA expression was examined by RT-PCR.
(D) NHEKs were treated with Poly I:C (10 μg/mL) for indicated times and harvested. Additionally, 50 ng/mL of EGF was used for stimulation as a positive control. GAPDH was used as a loading control. The experiment was analyzed by western blotting.
(E) NHEKs were pre-incubated with ERK inhibitor (PD98059 at 50 μM), p38 inhibitor (SB202190 at 5 μM), NF-κB inhibitors (parthenolide at 5 μM), or EGFR inhibitors (PD15035 5 μM and PD168393) for 30 min before stimulation with Poly I:C (10 μg/mL) for 8 h. IL-33 mRNA expression was examined by RT-PCR.
(F) TBK-1 inhibitor (SU6668) for 30 min before stimulation with Poly I:C (10 μg/mL) for 8 h. IL-33 mRNA expression was examined by RT-PCR.
(G) Hypothetic signaling pathways to induce IL-33 through TLR3 stimulation by viral infection.
All bars represent standard deviation (SD).
All results are expressed as the mean ± SD of 3 independent experiments.
Post-hoc test (Bonferroni/Dunn) was applied for statistical evaluation.

involved in anti-viral reactions, such as airway infection with influenza virus or parainfluenza virus, and cutaneous infection with herpes simplex virus [3]. Other studies reported that IL-33 from herpes simplex virus infection led to mast cell-mediated antiviral activation [4]. However, the source and mechanism of IL-33 induction remains unclear. Several reports indicate that Poly I:C stimulation caused IL-33 synthesis in several types of cell (Natarajan et al. [5], Zhang et al. [6], Talabot-Ayer et al. [7]), but there has been no report on normal human epidermal keratinocyte (NHEKs). In addition, it has not been clear if Poly I:C induces IL-33 at promoter level.

NHEKs from neonatal foreskin were purchased from GIBCO (Grand Island, NY, USA), which were stimulated with polyinosinic-polycytidylic acid [Poly I:C] (Novus Biologicals, Littleton, CO, USA), incubated for the indicated time periods, and harvested for experiments. Cells were treated with the following signaling inhibitors for at least 30 min before stimulation: PD98059 (50 μM, an inhibitor for ERK), SB202190 (5 μM, an inhibitor for p38), parthenolide (5 μM, an NF-κB inhibitor), PD153035, or PD168393 (5 μM, EGFR inhibitors), SU6668 (5 μM, TBK-1 inhibitor; Abcam, Cambridge, UK), and GM6001 (10 μM, TNFα-converting enzyme (TACE), also called as a disintegrin and metalloprotease (ADAM) 17 inhibitor). All signaling inhibitors were purchased from Calbiochem (San Diego, CA, USA). Total cellular RNA was isolated using TRIzol Reagent (Ambion, Foster City, CA, USA) and was reverse-transcribed using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Primers and probes for human IL-33

(Hs01125946_m1), IRF3 (Hs01547282_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems (Foster City, CA, USA). RT-PCR was performed on an Applied Biosystems StepOnePlus real-time PCR system using THUNDERBIRD Probe qPCR Mix (TOYOBO). Western blot was performed using anti-human IL-33 goat polyclonal antibody (R&D Systems), anti-IRF3 rabbit monoclonal antibody (Abcam), phospho-EGFR (Cell Signaling Technology, Danvers, MA, USA). Goat polyclonal Ab to GAPDH (Abcam) was used as the cytosol and nuclear internal controls. siRNA control or pre-designed siRNA for IRF3 were from Ambion. The cells were treated with siRNA using lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA). Three distinct exon1s of IL-33 was obtained as previously described [8]. FuGENE HD transfection reagent (Promega) was used. MTT assay was performed with Premix WST-1 Cell Proliferation Assay System (TaKaRa). Immunostaining of TLR3 and phospho-IRF3 was performed using the avidin-biotin–horseradish peroxidase (HRP) method (VECTASTAIN ABC Kit; Vector Laboratories, Burlingame, CA, USA). All data were presented as the mean ± SD. The paired student's *t*-test or post-hoc test (Bonferroni/Dunn) was performed utilizing Statcel4 software by OMS Publishing Inc. (Saitama Japan), to analyze differences between the data obtained from different experimental groups.

We previously identified and cloned IL-33 promoter region, which consisted of three independent promoters, e1aL, e1bL and e1cL [8]. Poly I:C induced IL-33 promoter activity in NHEKs, as well as IL-33 protein expression (Fig. 1A, B). IRF3 is a transcription factor activated by IFNs and TLRs, and has been shown to induce IL-33 in other cell types. Co-transfection of IRF3 expression vector with the IL-33 promoter vector resulted in activation of IL-33 promoter activity compared to co-transfection with the control vector in NHEKs (Fig. 1C), suggesting that IRF3 binds the IL-33 promoter region and induces its activity. Transfection of IRF3 siRNA effectively inhibited IL-33 mRNA induction by Poly I:C (Fig. 1D). It also suppressed induction of IL-33 protein by Poly I:C (Fig. 1E). Poly I:C, a double stranded RNA, signals through receptors such as TLR3, RIG1, and PKR3. Addition of TLR3 inhibitor suppressed the induction of the mRNA and protein levels of IL-33 by Poly I:C (Fig. 2A–B). TACE/ADAM17 is a metalloproteinase that processes membrane-bound pro-TNFα into soluble mature TNFα. It has also been reported to process membrane-bound pro-TGFα into its soluble active form. TACE inhibitor GM6001 blocked induction of IL-33 by Poly I:C (Fig. 2C) and phosphorylation of EGF (Tyr-845) was confirmed by stimulation with Poly I:C by western blotting (Fig. 2D). These results indicate that TACE/ADAM17-mediated cleavage of EGFR ligands, such as TGFα, could be involved in inducing IL-33 by Poly I:C. The above results demonstrated that EGFR was phosphorylated following stimulation with Poly I:C, suggesting that downstream signaling molecules, such as ERK, and p38 were involved. NFκB has been known to be activated downstream of TLR3. Inhibitors of ERK, p38, NF-κB, and EGFR phosphorylation all inhibited the induction of IL-33 mRNA by Poly I:C at 8 h after stimulation (Fig. 2E–F).

We have previously reported that IL-33 was expressed in herpes virus infection, but not in HPV infection [9]. We speculated that self RNA released from damaged cells in herpes virus infection induced IL-33. The above results indicated that IRF3 was involved in the induction of IL-33 in dsRNA-induced IL-33 expression. As we expected, phospho-IRF3 was stained in the nucleus of lesional keratinocytes and giant cells in herpes virus infection, but not in HPV infection (Supplementary Fig. 1), indicating that IRF3 was activated in herpes virus infection, but not in HPV infection, which could explain the difference in IL-33 expression in these two distinct cutaneous virus infections.

The results in our study indicate that following the stimulation of TLR3 by Poly I:C, several pathways are stimulated concurrently (Fig. 2G). The inhibition of each pathway abolished almost completely the induction of IL-33 by Poly I:C, suggesting that each pathway is not sufficient, but necessary for the induction of IL-33 in NHEKs.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.11.002.

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