

**Studies on the Pathophysiological Roles of Interleukin-33
in Canine Atopic Dermatitis**

(犬アトピー性皮膚炎の病態生理における
Interleukin-33 の役割に関する研究)

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The United Graduate School of Veterinary Sciences, Gifu University

(Gifu University)

ASAHINA, Ryota

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Abbreviations

ACTB: beta actin

AD: atopic dermatitis

cAD: canine atopic dermatitis

CCL: CC motif chemokine ligand

CCR4: CC chemokine receptor 4

Ct: Cycle threshold

CXCL10: CXC motif chemokine ligand 10

DAB: 3,3-diaminobenzidine

DPBS: Dulbecco's phosphate-buffered saline

FBS: fetal bovine serum

GATA3: GATA binding protein 3

GM-CSF: granulocyte-macrophage colony-stimulating factor

HDM: house dust mite

HEK: human embryonic kidney

HPRT1: hypoxanthine phosphoribosyl-transferase 1

HRP: horseradish peroxidase

IFN: interferon

IL: Interleukin

IgE: immunoglobulin E

IgG: immunoglobulin G

mAb: monoclonal antibody

NGS: normal goat serum

PBMCs: peripheral blood mononuclear cells

PBS: phosphate-buffered saline

rc: recombinant canine

rh: recombinant human

RPL32: ribosomal protein L32

RPS18: ribosomal protein S18

RT-qPCR: quantitative reverse transcription PCR

S100A8: S100 calcium-binding protein A8

SD: standard deviation

SDS: sodium dodecyl sulfate

ST2: suppression of tumorigenicity 2

STAT: signal transducer and activator of transcription

TARC: thymus and activation-regulated chemokine

TBST: Tris-HCl buffer saline with Tween 20

TCR: T cell receptor

TNF: tumor necrosis factor

TSLP: thymic stromal lymphopoietin

Th1: Type 1 helper T

Th2: Type 2 helper T

Th17: Type 17 helper T

General Introduction

Canine atopic dermatitis (cAD) is a chronic inflammatory skin disease that is commonly associated with immunoglobulin E (IgE) antibodies directed against environmental allergens such as house dust mite (HDM) [13]. Accumulated evidence has shown that cAD is characterized by a Type 2 helper T (Th2)-associated inflammatory disease, as well as human atopic dermatitis (AD) [16,31,32,43,50]. It is currently proposed that dysfunction of skin barriers may play roles in the pathogenesis of AD by facilitating penetration of antigens through the skin and consequently evoking Th2-associated inflammation [35]. In both human and canine AD, keratinocytes are implicated in the pathogenesis of AD by producing various soluble factors that induce Th2-associated inflammation, such as CC motif chemokine ligand 17 / thymus and activation-regulated chemokine (CCL17/TARC), thymic stromal lymphopoietin (TSLP), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [2]. Accordingly, the investigations focused on keratinocyte-derived cytokines may be able to provide new paradigms for biomarker and therapeutic strategy for human and canine AD.

Interleukin (IL)-33 is a novel member of epithelial cytokines, and is mainly expressed by keratinocytes in the skin [41,51]. In humans, IL-33 mediates its biological effects through the receptor suppression of tumorigenicity 2 (ST2), which is preferentially expressed on Th2 cells [25,51]. IL-33 has been shown to enhance the production of IL-5 and IL-13 from human and murine Th2 cells activated via T cell receptor (TCR) [23,51,54]. In the absence of TCR activation, IL-33 has also been

shown to enhance the production of IL-5 and IL-13 in Th2 cells activated by IL-2 [12,54]. Numerous studies indicate that these Th2 cytokines play a pivotal role in the pathogenesis of allergic inflammation and epidermal barrier defects [4,35]. Therefore, IL-33 has the ability to aggravate Th2-associated inflammation in the lesional skin of AD. Indeed, several studies on human AD revealed that the expression of IL-33 was up-regulated in keratinocytes of the lesional skin [18,48]. Moreover, AD-like lesions developed in transgenic mice overexpressing IL-33 in keratinocytes [17]. These findings indicate that IL-33 is a key keratinocyte-derived cytokine that promotes Th2-associated inflammation via Th2 cells in the pathogenesis of human AD. In dogs, previous studies reported that the transcription level of *il-33* was increased in the skin of sensitized dogs after exposure to HDM [44,49]. However, the involvement of IL-33 in the immunopathogenesis of cAD currently remains unclear.

In this doctoral dissertation, I carried out a series of studies to clarify the involvement and pathophysiological roles of IL-33 in cAD. The present study consists of three chapters as follows.

In chapter 1, the transcription of *st2* and the Th2 cytokine profile after stimulation with IL-33 in canine Th2 cells were investigated to reveal the effects of IL-33.

In chapter 2, the transcription and expression of IL-33 in the lesional skin of cAD were examined to demonstrate a relationship with immunopathogenesis.

In chapter 3, the transcription level of *il-33* was quantified in cultured canine

keratinocytes stimulated with IFN-gamma or IL-17A to identify the inducing factors of IL-33.

Chapter 1

Transcriptional analysis of the IL-33 receptor (ST2) and its effects on canine Th2 cells: a preliminary study

1. 1. Introduction

IL-33, a novel member of epithelial cells-derived cytokines [41], is released during tissue damage and necrosis and functions as an endogenous danger signal, also known as ‘alarmin’ [6]. IL-33 mediates its biological effects through the IL-33 receptor (ST2), which is preferentially expressed on human and murine Th2 cells [25,29]. *In vitro*, IL-33 has been shown to enhance the production of Th2 cytokines including IL-5 and IL-13 in human and murine Th2 cells [51,54]. Furthermore, IL-33 promoted the differentiation of human and murine Th2 cells from naïve T cells *in vitro*, independently of IL-4 [24]. IL-33 was also found to function as a chemoattractant for human Th2 cells *in vitro* [22]. The administration of IL-33 to mice was previously shown to increase the expression of Th2 cytokines in multiple tissues, elevate serum IgE levels and cause eosinophilia [51]. Additionally, a subcutaneous injection of IL-33 induced cutaneous inflammatory responses with increased serum IL-5 and IL-13 concentrations in mice, but had no effects in ST2-deficient mice [15]. Based on these findings, IL-33 has the ability to aggravate Th2-associated inflammation via ST2 on Th2 cells.

In humans, genetic polymorphisms in the ST2 region have been associated with AD, which is a common Th2-associated inflammatory disease [53]. The transcription level of *il-33* in the lesional skin of human AD was found to be higher than that in the non-lesional skin [3]. Additionally, a number of ST2-expressing cells were shown to infiltrate the dermis and epidermis of human AD [48]. Immunohistochemical

analyses identified keratinocytes as the major cellular source of IL-33 in the lesional skin of human AD [38,48], while IL-33 may be induced in human keratinocytes by several factors, such as pro-inflammatory cytokines, allergens and bacteria [48]. Moreover, AD-like lesions developed in transgenic mice expressing IL-33 driven by a keratin 14 promoter in keratinocytes [17]. IL-33-deficient mice and ST2-deficient mice showed the suppression of Th2-associated inflammation in a mouse model of experimental AD [27]. These findings suggest that IL-33 acts as a key mediator to promote Th2-associated inflammation in the pathogenesis of human AD. Therefore, the IL-33/ST2 pathway is a potential therapeutic target for human AD.

In dogs, cAD is generally regarded as a Th2-associated disease, similar to human AD, because previous studies have demonstrated the involvement of Th2 cytokines in its pathogenesis [43,50]. Several studies revealed that the transcription of *il-33* was increased in the skin of dogs sensitized to HDM antigens, a major environmental allergen in cAD [44,49]. Consequently, IL-33 may be involved in the pathogenesis of cAD, similar to human AD. Thus, IL-33 has the potential to induce the production of Th2 cytokines in canine Th2 cells. However, the effects of IL-33 and the expression of ST2 on canine Th2 cells have not yet been investigated.

In the present study, I investigated the transcription of *st2* in canine T cell subsets and the Th2 cytokine profile in canine Th2 cells stimulated with IL-33. The present study was conducted as a preliminary study due to the small sample size.

1. 2. Materials and Methods

1. 2. 1. Animals and cell sorting

Three healthy dogs kept for experimental purposes, a female and two male beagle dogs aged between 1.5 and 4.3 years (average 2.7 years) without any clinical signs of diseases, were used. Forty millilitre samples of heparinized whole blood were obtained from each dog and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with LymphoprepTM (Axis-Shield, Oslo, Norway) according to the manufacturer's instructions. All procedures were approved by the Institutional Animal Care and Use Committee of Gifu University (#16082). In the sorting of each T cell subset, PBMCs were incubated with antibodies against CD4, CD8 and CC chemokine receptor 4 (CCR4), as a cell surface antigen of helper T cells, cytotoxic T cells and Th2 cells, respectively, at 4°C for 30 min. Appropriate isotype controls were used for each sample. The antibodies used are listed in Table 1-1 [11,31]. Cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS containing 1% fetal bovine serum (FBS). The fraction of lymphocytes was initially gated, followed by the sorting of CD4⁺, CD8⁺, CCR4⁺CD4⁺ and CCR4⁻CD4⁺ lymphocytes using a SH800 Cell Sorter (Sony Biotechnology Inc., Tokyo, Japan) equipped with SH800 software (Sony Biotechnology Inc.). The proportions of sorted cells were analysed to verify successful sorting.

1. 2. 2. Cell culture and stimulation

Sorted CCR4⁺CD4⁺ cells (Th2 cells) were cultured in 24-well flat-bottomed plates (NUNC, Roskilde, Denmark) at a concentration of 5×10^5 cells per well in 1 mL of RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS. In the transcription analysis of unstimulated cells, total RNA was extracted promptly after cell sorting. The remaining cells were cultured for 12 h with recombinant canine (rc) IL-33 (20 ng/mL [51]; Sino Biological Inc., Beijing, China) and/or recombinant human (rh) IL-2 (5 ng/mL [7,20,26]; PeproTech, Rocky Hill, NJ, USA), which is known as a cooperative signal of IL-33 [12,54], in RPMI 1640 medium supplemented with 10% FBS at 37°C in air supplemented with 5% CO₂. The transcription levels of cytokines were evaluated among various concentrations of rcIL-33 (0, 0.2, 2, 20 and 200 ng/mL [17,27]) in the presence of rhIL-2 (5 ng/mL).

1. 2. 3. Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from cell pellets using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Genomic DNA was removed from samples with a TURBO DNA-free Kit (Applied Biosystems, Foster City, CA, USA) and then stored at -80°C. Total RNA was reverse transcribed to cDNA using the PrimeScriptTM RT Reagent Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The transcription of *st2* in each T cell

subset was quantified by two-step RT-PCR using the Thermal Cycler Dice Real Time System TP800 (Takara Bio Inc.). Similarly, the transcription of *il-4*, *il-5*, *il-13* and *il-31* in stimulated and unstimulated Th2 cells was quantified. The primer pairs used in this study are listed in Table 1-2. The specificities of the primers were confirmed to amplify each target mRNA by a sequential analysis of PCR products using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed using SYBR Premix Ex TaqTM II (Takara Bio Inc.). In order to ensure accurate quantification, three reference genes including ribosomal protein S18 (RPS18), beta actin (ACTB) and hypoxanthine phosphoribosyl-transferase 1 (HPRT1) were selected by GeNorm (<https://genorm.cmgg.be>) among eight candidate reference genes (GAPDH, ACTB, RPS18, CG14980, B2M, HPRT1, SDHA and TBP). Amplification consisted of a first period of activation (95°C for 10 s) followed by 47 cycles of a PCR reaction (95°C for 5 s and 60°C for 30 s) and dissociation (95°C for 15 s, 60°C for 30 s and 95°C for 15 s). Each PCR reaction included samples without reverse transcription, which were not amplified in the present study. Cycle threshold (Ct) values, which indicate the point at which the threshold intersects with the amplification curve of the PCR reaction, were obtained using software (Thermal Cycler Dice Real Time System Multiplate RQ software, Version 3.00; Takara Bio Inc.). Each transcription level (relative quantity) was quantified using the comparative Ct ($2^{-\Delta\Delta C_t}$) method, by which the transcription levels of the target genes in each sample were represented as an *n*-fold difference from that in the calibrator sample. The Ct values of the target genes were

subtracted from the geometric mean of the Ct values of the three reference genes (ΔCt) [62]. All samples were examined in duplicate, and the mean value of ΔCT was calculated. The relative quantity of target genes was calculated by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{samples}} - \Delta Ct_{\text{calibrator samples}}$, using CD4⁺ helper T cells for the transcription of *st2* or rhIL-2-stimulated cells for the transcription of *il-4*, *il-5*, *il-13* and *il-31* as the calibrator sample. Finally, the mean relative quantity was described by the ratio of the samples to the calibrator samples with the standard deviation (SD) of three dogs.

1. 2. 4. *Statistical analysis*

The transcription levels of each gene were statistically analysed by the Mann-Whitney U test for the comparison of two samples or Dunn's test for the comparison of more than two samples. A value of $P < 0.05$ was considered to be significant. Statistical analyses were performed using the JMP 10.0 program (SAS Institute, Cary, NC, USA).

1. 3. Results

1. 3. 1. *Transcription levels of st2 in PBMCs*

The purity of the sorted cells was no less than 94% (data not shown). The transcription of *st2* in CD4⁺ helper T cells was significantly higher than that in CD8⁺ cytotoxic T cells ($P < 0.05$; Figure 1-1a). The relative quantity of *st2* in CCR4⁺CD4⁺ lymphocytes was approximately 14-fold higher than that in CD4⁺ helper T cells ($p = 0.037$; Figure 1-1b). In contrast, the transcription of *st2* in CCR4⁻CD4⁺ lymphocytes, which is more likely to include mostly Type 1 helper T (Th1) cells [16], was under the detection limit (Figure 1-1b). Statistical analyses were not performed because the transcription of *st2* was not observed in CCR4⁻CD4⁺ lymphocytes.

1. 3. 2. *Transcription levels of il-4, il-5, il-13 and il-31 in Th2 cells*

The transcription levels of *il-5* and *il-13* in Th2 cells were below the detection limit in unstimulated samples, but peaked following the stimulation with rcIL-33 and rhIL-2 (Figure 1-2a, b). Notably, the transcription level of *il-13* in cells stimulated with rcIL-33 and rhIL-2 was significantly (approximately 14-fold) higher than that in cells stimulated with rhIL-2 only ($P < 0.05$; Figure 1-2b). Additionally, the transcription of *il-13*, in the stimulation with rcIL-33 only, was below the detection limit (Figure 1-2b).

The transcription levels of *il-5* in cells stimulated with rcIL-33 and rhIL-2 were significantly higher than those in cells stimulated with rcIL-33 only ($P < 0.05$), even though cells stimulated with rhIL-2 only were able to transcribe the gene of *il-5* (Figure 1-2a). Statistical analyses were performed for each gene in samples in which transcription was detected. The transcription levels of *il-4* and *il-31* were below the detection limit for all culture conditions (data not shown). Since the transcription of *il-5* and *il-13* was induced by a co-stimulation with rcIL-33 and rhIL-2, cells were stimulated with various concentrations of rcIL-33 in the presence of rhIL-2. The transcription levels of *il-5* and *il-13* in rcIL-33-stimulated cells at 200 ng/mL were significantly higher than those in unstimulated cells (Figure 1-3a, b).

1. 4. Discussion

In previous studies using human and murine cells, polarized Th2 cells that differentiated from naïve T cells *in vitro* were used to evaluate the expression of ST2 and effects of IL-33 on Th2 cells [12,25,51,54]; however, these methods have never been established in dogs. One study demonstrated that CCR4⁺CD4⁺ cells sorted from healthy dogs have the ability to produce Th2 cytokines including IL-4 and IL-13 in response to phorbol 12-myristate 13-acetate and calcium ionophores [16]. Consequently, CCR4⁺CD4⁺ cells were used as canine Th2 cells in the present study.

A flow cytometric analysis of ST2 is commonly used in investigations on the target cells of IL-33 in humans and mice [29,65]; however, there is currently no antibody available against ST2 in dogs. Hence, a transcriptional analysis of *st2* was performed in the present study. My results revealed that the transcription of *st2* was the highest in CCR4⁺CD4⁺ lymphocytes (Th2 cells) among the cells examined. Thus, it is suggested that canine Th2 cells are the main cells expressing ST2 among T cell subsets, which is similar to previous findings obtained from humans and mice [12,25,28,65]. Since human ST2 is preferentially expressed on Th2 cells, and not naïve T cells, Th1 cells, Type 17 helper T (Th17) cells or regulatory T cells in CD4⁺ T cells, ST2 may be used as a stable cell surface marker of human Th2 cells [12,25]. However, a flow cytometric analysis revealed that human ST2 is also expressed on innate immune cells including mast cells, basophils, eosinophils, group 2 innate lymphoid cells, natural killer

cells and natural killer T cells [45]. These results prompted us to investigate the cellular targets of IL-33 in the innate cells of dogs in more detail.

IL-33 was previously reported to enhance the production of IL-5 and IL-13 from human and murine Th2 cells activated via TCR-dependent antigens [23,51,54]. Furthermore, in the absence of TCR activation, human and murine Th2 cells stimulated with IL-33 may produce IL-5 and IL-13 by the addition of signal transducer and activator of transcription (STAT)5 activators such as IL-2, IL-7 and TSLP [12,54]. The concerted effects of IL-33 and STAT5 activators up-regulated the re-expression of GATA binding protein 3 (GATA3) in resting murine Th2 cells, while GATA3 was shown to regulate the expression of ST2 on murine Th2 cells [12]. This TCR-independent production of Th2 cytokines by human and murine Th2 cells in response to IL-33 and STAT5 activators provides a mechanism for the innate activation of Th2 cells in humans and mice [12]. Similarly, my results demonstrated that IL-33 enhanced the transcription of *il-5* and *il-13* in canine Th2 cells in the presence of IL-2, suggesting that IL-2 acted as a cooperative signal of IL-33. In human AD, IL-33 is most likely released from keratinocytes by mechanical injury due to scratching behavior [57]. Additionally, a number of ST2-expressing cells were shown to infiltrate the lesional skin of human AD. Thus, it is considered that IL-33 acts as a mediator to promote Th2-associated inflammation via Th2 cells in human AD. Previous studies revealed that an exposure to HDM antigens induced the transcription of *il-33* in the skin of the sensitized dogs [44,49]. Moreover, it is shown that the increased transcription of the STAT5 activator

including *il-2* in the lesional skin of cAD [43]. These findings suggest that IL-33 aggravates Th2-associated inflammation in cooperation with IL-2 through the production of IL-5 and IL-13 in Th2 cells in the lesional skin of cAD.

My results demonstrated that IL-33 had no effect on the production of IL-4 or IL-31 in canine Th2 cells, similar to humans and mice [12,23,51,55]. A previous study reported that the production of IL-4 in murine Th2 cells fully depended on the nuclear factor of activated T cells pathway, whereas that of IL-5 and IL-13 by IL-33 was mainly mediated by p38 and nuclear factor-kappa B [12]. Furthermore, a co-stimulation with STAT6, but not STAT5, was essential for the production of IL-31 in human Th2 cells activated by IL-33 [34]. Therefore, I speculate that the reason why canine Th2 cells did not produce IL-4 or IL-31 in the present study is due to differences in the signaling pathways required for the production of each Th2 cytokine. In order to demonstrate this hypothesis, the signaling pathways for the production of each Th2 cytokine in canine Th2 cells need to be investigated in future studies.

In conclusion, this is the first study to show the effects of IL-33 on canine Th2 cells. My results suggest that IL-33 enhances Th2-associated inflammation in cooperation with IL-2 by inducing IL-5 and IL-13 in canine Th2 cells. Additional studies will be required to investigate whether IL-33 contributes to the pathogenesis of cAD.

1. 5. Table and Figure

Table 1-1. Antibodies used in cell sorting.

Antigen	Clone	Isotype	Concentration	Conjugation	Source	Reference
anti-dog CD4	YKIX302.9	rat IgG2a	1:50	FITC	Bio-Rad*	Gorman SD et al. ¹¹
anti-dog CD8	YCATE55.9	rat IgG1	1:10	ALEXA FLUOR 647	Bio-Rad*	Gorman SD et al. ¹¹
anti-human CCR4	1G1	mouse IgG1	1:10	PE	BD Pharmingen†	Maeda S et al. ³¹
rat IgG2a			1:50	FITC	Bio-Rad*	
rat IgG1			1:10	ALEXA FLUOR 647	Bio-Rad*	
mouse IgG1			1:10	PE	BD Pharmingen†	

FITC, fluorescein isothiocyanate; PE, phycoerythrin. *Hercules, CA, USA. †San Diego, CA, USA.

Table 1-2. Sequences of primers for RT-qPCR including receptor, cytokine, and reference genes.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
IL-4	TCTGCTTACTAGCACTCACCAGCAC	GACAGTCAGCTCCATGCACGA
IL-5	GCCTATGTTTCTGCCTTTGC	GGTCCCATCGCCTATCA
IL-13	CTGGTCAACATCACCCAGAATCA	GCACAGTGCTTTCAGCATCCTC
IL-31	TGTGCCTGCAGATACTTTTGA	GGTTCGACCAGATAGCCTTG
ST2	AATTACAGCGTGACAGCAACC	CCAAAGCAAGCAGAGCAGAG
ACTB	CCAGCAAGGATGAAGATCAAG	TCTGCTGGAAGGTGGACAG
HPRT1	CACTGGGAAAACAATGCAGA	ACAAAGTCAGGTTTATAGCCAACA
RPS18	TGCTCATGTGGTATTGAGGAA	TCTTATACTGGCGTGGATTCTG

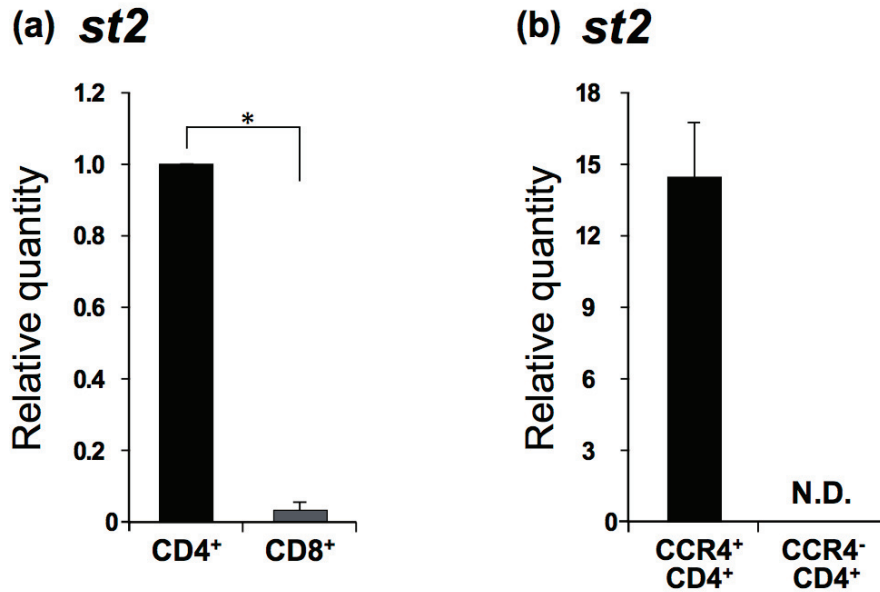


Figure 1-1. Transcription analysis of *st2* (a) in CD4⁺ helper T cells and CD8⁺ cytotoxic T cells, and (b) in CCR4⁺CD4⁺ (Th2 cells) or CCR4⁻CD4⁺ lymphocytes. Three genes (RPS18, ACTB and HPRT1) were used as reference genes. The relative quantity of *st2* in each cellular subset represents an *n*-fold difference from that in CD4⁺ helper T cells. Results represent the means \pm SD of three different dogs. **P* < 0.05 by the Mann-Whitney U test; N.D., not detected.

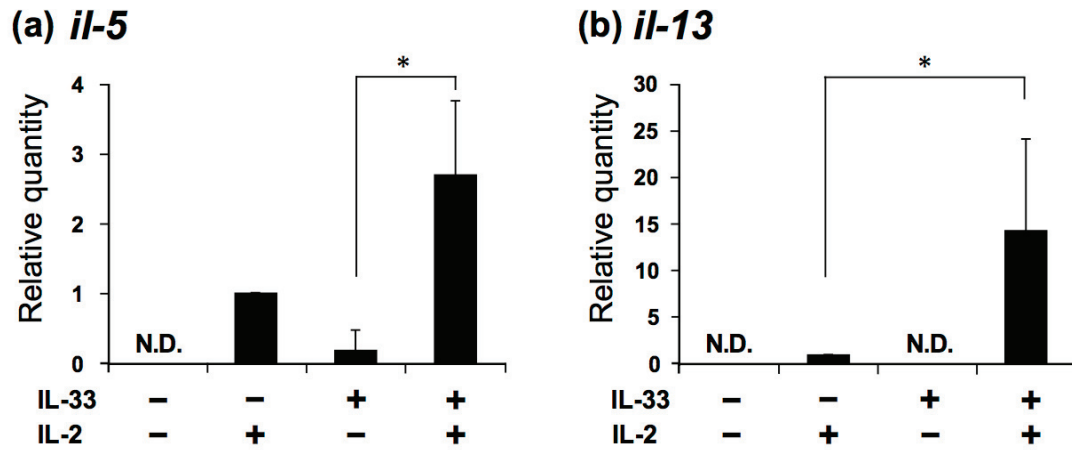


Figure 1-2. Transcription analysis of (a) *il-5* and (b) *il-13* in canine Th2 cells stimulated with recombinant canine IL-33 (20 ng/mL) and/or recombinant human (rh) IL-2 (5 ng/mL). Three genes (RPS18, ACTB and HPRT1) were used as reference genes. The relative quantity of each gene in stimulated cells represents an *n*-fold difference from that in rhIL-2-stimulated cells because of the absence of detectable transcription in unstimulated cells. Results represent the means \pm SD of three independent experiments. * $P < 0.05$ by (a) Dunn's or (b) the Mann-Whitney U test; N.D., not detected.

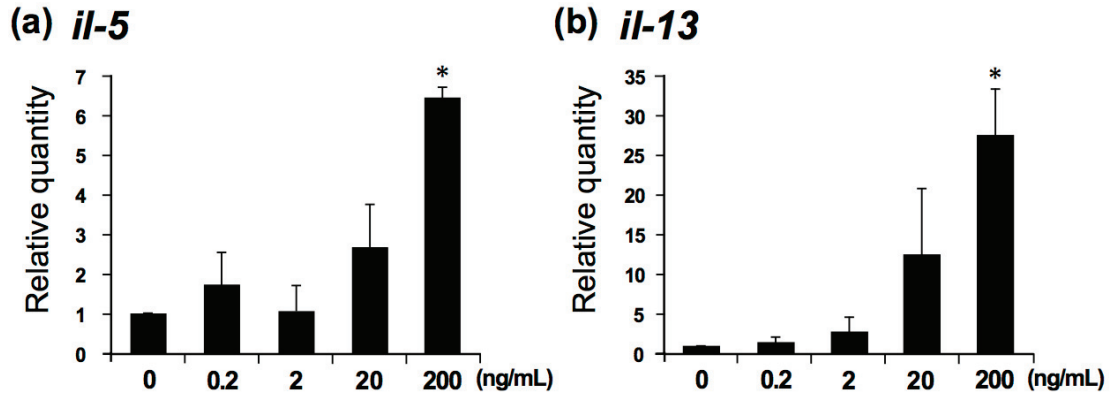


Figure 1-3. Transcription analysis of (a) *il-5* and (b) *il-13* in canine Th2 cells stimulated with various concentrations of recombinant canine (rc) IL-33 in the presence of recombinant human (rh) IL-2. Three genes (RPS18, ACTB and HPRT1) were used as reference genes. The relative quantity of each gene in rcIL-33-stimulated cells represents an *n*-fold difference from that in rhIL-2-stimulated cells. Results represent the means \pm SD of three independent experiments. The significance of differences ($*P < 0.05$) between cultures with and without rcIL-33 was assessed with Dunn's test.

Chapter 2

Expression of IL-33 in lesional skin of canine atopic dermatitis

2. 1. Introduction

Canine AD is a Th2-associated inflammatory disease, similar to human AD [16,31,32,50]. In human and canine AD, keratinocytes have been shown to produce various soluble factors that induce Th2-associated inflammation, suggesting that keratinocyte-derived cytokines play a pivotal role in the pathogenesis of human and canine AD [2]. IL-33, a novel keratinocyte-derived cytokine [41], is released in response to tissue damage, and then binds to its receptor (ST2) [36]. In humans and mice, ST2 is preferentially expressed on Th2 cells [25]. IL-33 has been shown to enhance the production of Th2 cytokines including IL-5 and IL-13 in Th2 cells [51,54]. The administration of recombinant IL-33 [15] or transgenic expression of IL-33 in keratinocytes [17] induced cutaneous inflammation with the increased expression of Th2 cytokines in the serum and skin. Based on these findings, it is considered that IL-33 has the ability to aggravate Th2-associated inflammation via ST2 on Th2 cells.

Several studies revealed the up-regulation of IL-33 expression and infiltration of ST2-expressing cells in the lesional skin of human AD [3,48]. Immunohistochemical analyses demonstrated that keratinocytes were the major cellular source of IL-33 production in the lesional skin of human AD [18,38]. Additionally, serum IL-33 levels were significantly higher in AD patients than in healthy controls [18,57]. Serum IL-33 levels also correlated with severity, excoriation and xerosis scores, suggesting that scratching behavior promotes the release of IL-33

from the skin [57]. These findings indicate that IL-33 acts as a key keratinocyte-derived cytokine that promotes Th2-associated inflammation in the pathogenesis of human AD. Therefore, IL-33 has the potential as a biomarker and therapeutic target for human AD.

In this chapter 1, I demonstrated that canine Th2 cells were the main cells transcribing *st2* among T cell subsets. I also indicated that IL-33 enhanced the transcription of *il-5* and *il-13* in canine Th2 cells activated by IL-2. These findings suggest that canine IL-33 enhances Th2-associated inflammation via Th2 cells, similar to humans and mice. Previous studies revealed that the transcription of *il-33* was increased in the acute skin lesions of experimentally sensitized dogs with HDM antigen [44,49]. Consequently, IL-33 appears to contribute to Th2-associated inflammation in the lesional skin of cAD, similar to human AD. However, the involvement of IL-33 in the pathogenesis of cAD currently remains unclear.

In the present study, I investigated the transcription and expression of IL-33 in chronic lesional skin of spontaneous cAD in order to demonstrate a relationship with immunopathogenesis.

2. 2. Materials and Methods

2. 2. 1. Animals and sample collection

Eight dogs with spontaneous cAD were recruited for this study. The diagnosis of cAD was based on the combination of a compatible history and the fulfillment of five published criteria [9]. Other pruritic skin diseases, such as flea allergy dermatitis, scabies, demodicosis, pyoderma and *Malassezia* dermatitis, were excluded based on routine dermatological examinations and therapeutic trials. In cases exhibiting year-round clinical signs, an elimination diet trial for a minimum of eight weeks was performed in order to rule out a cutaneous adverse food reaction. Anti-inflammatory agents including glucocorticoids, cyclosporin, and oclacitinib were withdrawn for at least 2 weeks prior to skin biopsy collection. The breeds, ages, sex, and lesions for skin biopsy are listed in Table 2-1. Five healthy dogs kept for experimental purposes, two spayed female and three male beagle dogs aged between 5.5 and 7.6 years (average 6.6 years) without any clinical signs of diseases, were used as control samples.

Skin biopsy specimens were obtained from chronic lesional skin showing lichenification or pigmentation. Each specimen was divided into two pieces. One half was fixed in 10% formalin, and processed under a paraffin-embedding protocol for immunohistochemical staining. The other half was immediately submerged in

RNAlater (QIAGEN, Valencia, CA, USA) and stored at -30°C until the extraction of total RNA. All procedures were approved by the Institutional Animal Care and Use and Clinical Ethics Committees of Gifu University (#15001, #E15001).

2. 2. 2. *RT-qPCR*

The steps from total RNA extraction to RT-PCR were carried out as described in this chapter 1. In order to ensure accurate quantification, three reference genes including CG14980, ribosomal protein L32 (RPL32) and RPS18 were selected by GeNorm (<https://genorm.cmgg.be>) among eight candidate reference genes (B2M, CG14980, GAPDH, HPRT1, RPL13A, RPL32, RPS18, and SDHA) [47]. The primer pairs used in the present study are listed in Table 2-2. The specificities of these primers were confirmed to amplify each target mRNA by a sequential analysis of PCR products using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Amplification consisted of a first period of activation (95°C for 10 s) followed by 40 cycles of a PCR reaction (95°C for 5 s and 60°C for 30 s) and dissociation (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s). Each PCR reaction included samples without reverse transcription, which were not amplified in the present study. Ct values, which indicate the point at which the threshold intersects with the amplification curve of the PCR reaction, were obtained using software (Thermal Cycler Dice Real Time System Multiplate RQ software, Version 3.00; Takara Bio Inc.).

The Ct values of *il-33* were subtracted from the geometric mean of the Ct values of the three reference genes (ΔCt) [62]. All samples were examined in duplicate, and the mean value of ΔCT was calculated. The transcription level of *il-33* was calculated by $2^{-\Delta\text{Ct}}$, resulting in the evaluation of samples as *n*-fold differences from the mean value of three reference genes.

2. 2. 3. *Transfection of canine IL-33 into human embryonic kidney (HEK) 293A cells*

HEK293A cells (Invitrogen, Carlsbad, CA, U.S.A.) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS at 37°C in an atmosphere of air containing 5% CO₂. A mammalian expression plasmid vector encoding canine full-length IL-33 at the C terminus (Sino Biological Inc., Beijing, China) was transfected into HEK293A cells using Lipofectamine™ 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfected cells were then used in immunoblotting and immunocytochemistry to confirm the cross-reactivity of the anti-human IL-33 monoclonal antibody (mAb) to canine IL-33 at 48 h post-transfection.

2. 2. 4. *Immunoblotting*

Transfected HEK293A cells were collected by scuffing and incubating in TNG-T (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 10% glycerol, and 1% Triton X-100) with protease inhibitor cocktail (Complete mini, Roche, Basel, Switzerland) at 4°C for 1 h. After centrifugation at 1000×g at 4°C for 5 min, supernatants were obtained. Protein concentrations were measured using Bradford's assay (Bio-Rad, Hercules, U.S.A.). Equal amounts of protein samples were mixed with 4% sodium dodecyl sulfate (SDS) and boiled at 95°C for 5 min. Protein samples were separated on 15% SDS-PAGE gels (Supersep Ace, Wako, Osaka, Japan) and transferred to polyvinylidene difluoride membranes (Immobilon-P membrane, Merck Millipore, Darmstadt, Germany). Non-transfected HEK293A cells and recombinant canine IL-33 (Sino Biological Inc.), which is the processed form of IL-33 cleaved by caspase-1, were used as the negative control and positive control, respectively. Membranes were blocked with 3% skim milk in Tris-HCl buffer saline with 0.1% Tween 20 (TBS-T) at room temperature for 1 h. Immunoblotting was conducted using a mouse monoclonal anti-human IL-33 antibody (clone Nussy-1; Enzo Life Sciences, Farmingdale, NY, USA; dilution 1:1000) in an incubation at 4°C overnight. After washing three times with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch, Pennsylvania, U.S.A., dilution 1:2500) at room temperature for 1 h. Immunoreactivity was detected by chemiluminescence (Chemi-Lumi One Super, Nacalai Tesque, Kyoto, Japan).

2. 2. 5. *Immunohistochemistry*

Transfected HEK293A cells were fixed for 10 min with 4% paraformaldehyde, and permeabilized with 0.25% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) in PBS at room temperature for 10 min. Endogenous peroxidase was blocked with 0.3% H₂O₂ in methanol at room temperature for 20 min. Non-specific reactions were blocked with 10% normal goat serum (NGS) in PBS at room temperature for 30 min. Cells were reacted with anti-human IL-33 mAb (Nessy-1, dilution 1:100) or an appropriate isotype control (normal mouse IgG1; BD Pharmingen, San Diego, CA, USA; dilution 1:100) in 10% NGS in PBS at 4°C overnight. HRP-conjugated goat anti-mouse IgG (EnVision™; Dako, Glostrup, Denmark, pre-diluted) was added and incubated at room temperature for 30 min as secondary antibodies. Peroxidase labeling was visualized with 3,3-diaminobenzidine (DAB) (Dako). Cells were counterstained with hematoxylin. Immunohistochemical staining was performed on the lesional skin of seven dogs with cAD and the normal skin of five healthy dogs. Formalin-fixed paraffin-embedded tissue sections were deparaffinized in xylene and ethanol. Epitope retrieval was performed with an autoclave (121°C, 15 min) in citrate buffer solution (Target Retrieval Solution, citrate pH 6, Dako). In order to prevent a non-specific reaction, endogenous peroxidase activity was quenched using 0.3% H₂O₂ in methanol for 20 min, and tissue sections were incubated in 10% NGS in

PBS at room temperature for 1 h. Sections were incubated with anti-human IL-33 mAb (Nessy-1, dilution 1:100) or an appropriate isotype control (normal mouse IgG1; BD Pharmingen; dilution 1:100) at 4°C overnight. The secondary antibody used was HRP-conjugated goat anti-mouse IgG (EnVision™; Dako) at room temperature for 30 min. After the reaction with DAB, sections were counterstained with hematoxylin.

2. 2. 6. *Statistical analysis*

The Mann–Whitney U test was used for comparisons of the transcription levels of *il-33* between the lesional skin from dogs with cAD ($n = 8$) and the normal skin from healthy dogs ($n = 5$). A value of $P < 0.05$ was considered to be significant. Statistical analyses were performed using the JMP 10.0 program (SAS Institute, Cary, NC, USA).

2. 3. Results

2. 3. 1. *Transcription level of il-33 in the lesional skin of cAD*

The transcription of *il-33* was detected in all skin samples by RT-qPCR. The transcription level of *il-33* in the lesional skin of cAD was significantly higher than that in the normal skin of dogs ($p = 0.019$; Figure 2-1).

2. 3. 2. *Cross-reactivity of anti-human IL-33 mAb to canine IL-33*

A single band near 30 kDa was detected and corresponded to the expected molecular weight of the full-length canine IL-33 protein in canine IL-33-transfected cells, but not in non-transfected cells (Figure 2-2a). In addition, a single band near 18 kDa was detected and corresponded to the expected molecular weight of the processed canine IL-33 protein, which is the cleaved N-terminal domain related to nuclear localization, in recombinant canine IL-33 (Figure 2-2a). Immunocytochemical analyses revealed that canine IL-33-transfected cells were positively stained with anti-human IL-33 mAb, and negatively stained with isotype control (Figure 2-2b). Non-transfected cells were negatively stained with anti-human IL-33 mAb (Figure 2-2b). The efficiency of transfection was approximately 50%.

2. 3. 3. *Expression of IL-33 in the lesional skin of cAD*

Immunohistochemical analyses revealed that keratinocytes in the stratum spinosum positively stained for canine IL-33 in all lesional skin samples of cAD, whereas no keratinocytes were stained in any of the normal skin samples (Figure 2-3).

2. 4. Discussion

In the present study, I found that the transcription level of *il-33* in the chronic lesional skin of cAD was higher than that in the normal skin of dogs. Previous studies using an experimental model of cAD reported that the transcription of *il-33* was increased in the acute skin lesions [44,49]. Thus, IL-33 is likely to contribute to both acute and chronic skin inflammation in the pathogenesis of cAD. Interestingly, my results suggested that the transcription level of *il-33* is remarkably high in skin lesions with excoriation. In human AD, serum IL-33 levels were most correlated with excoriation score [57]. Furthermore, the expression of IL-33 was increased in the skin of mice after tape stripping, which was used to mimic mechanical skin injury [39]. These findings suggest scratch injury is likely to induce a mechanical destruction releasing IL-33.

In this chapter, keratinocytes were found to be the major cellular source of IL-33 production in the lesional skin of cAD. My results showed that IL-33 was most likely to express in the nuclei of keratinocytes, as reported in human AD [3,18,38,48]. In humans, it is demonstrated that IL-33 translocates to the nucleus by nuclear localization sequence within its N-terminal domain after synthesis in the cytoplasm [5]. These results indicate that keratinocyte-derived IL-33 is involved in the immunopathogenesis of cAD. My results of this chapter 1 suggest that IL-33 has the ability to aggravate allergic inflammation via Th2 cells. Prospective studies that

evaluate the relationship between keratinocyte-derived IL-33 and the severity scores should be conducted.

A previous study reported that there are strong species differences in the expression and regulation of IL-33 in keratinocytes [56]. Murine IL-33 is known to be constitutively expressed in keratinocytes as a nuclear protein and rapidly released during inflammation [56]. In contrast, human and porcine IL-33 were weakly expressed or absent in the keratinocytes of normal skin and induced during inflammation [56]. My results showed that canine IL-33 was not expressed in the keratinocytes of normal skin of healthy dogs, suggesting that the regulation of IL-33 in dogs was similar to that in humans and pigs. However, the mechanism responsible for these species differences currently remains unclear.

In this chapter, there was a limitation that several factors including age, sex and breeds are not matched between dogs with cAD and healthy dogs. Therefore, it is necessary to investigate whether these factors are unaffected the transcription and expression of IL-33 in future studies.

In conclusion, this study is the first to investigate the expression of IL-33 in the chronic skin lesions of dogs with cAD. I demonstrated increases in the transcription of *il-33* and the selective expression of IL-33 by keratinocytes in the lesional skin of cAD. These results indicate that IL-33 is involved in the immunopathogenesis of cAD, similar to human AD.

2. 5. Table and Figure

Table 2-1. Clinical cases in the present study.

No.	Breed	Age (years)	Sex	Lesions for skin biopsy					
				Region	Erythema	Lichenification	Pigmentation	Excoriation	Alopecia
1	Shiba Inu	7.6	F	Axillae	+	+	-	+	+
2	Shiba Inu	8	F	Thorax	+	+	+	+	+
3	Shiba Inu	11	F	Thorax	+	+	+	+	+
4	Shiba Inu	12.9	SF	Axillae	+	+	+	-	+
5	Shiba Inu	14.6	SF	Axillae	+	+	+	-	+
6	Kishu Inu	11	SF	Inguinal area	+	+	+	-	+
7	Mongrel	10.3	F	Inguinal area	+	+	+	-	+
8	Mongrel	13.9	SF	Axillae	+	+	+	-	+

F: female, SF: spayed female

Table 2-2. Sequences of primers for RT-qPCR including cytokine and reference genes.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
IL-33	GTACTTTATGCAACTGCGTTCTGG	CAGACATTGCTTTCTGCACTTTTC
CG14980	GCAGGAAGGGATTCTCCAG	GGTCCAGTAAGAAATCTTCATAA
RPL32	TGGTTACAGGAGCAACAAGAAA	GCACATCAGCAGCACTTCA
RPS18	TGCTCATGTGGTATTGAGGAA	TCTTATACTGGCGTGGATTCTG

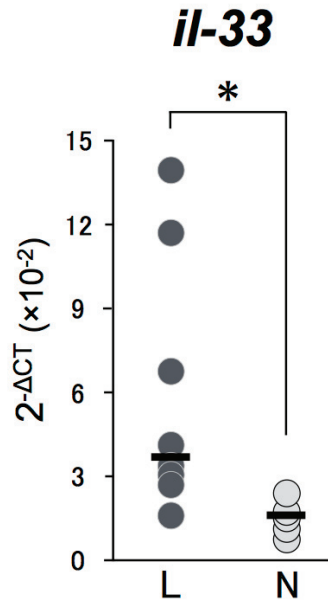


Figure 2-1. Transcription levels of *il-33* in the lesional skin of cAD (L, $n = 8$) and normal skin of healthy dogs (N, $n = 5$). The transcription levels of *il-33* were calculated by $2^{-\Delta C_t}$, resulting in the evaluation of samples as n -fold differences relative to three reference genes (CG14980, RPL32, and RPS18). Horizontal lines correspond to the median of the transcription levels of *il-33* in each group. $*P < 0.05$ by the Mann-Whitney U test.

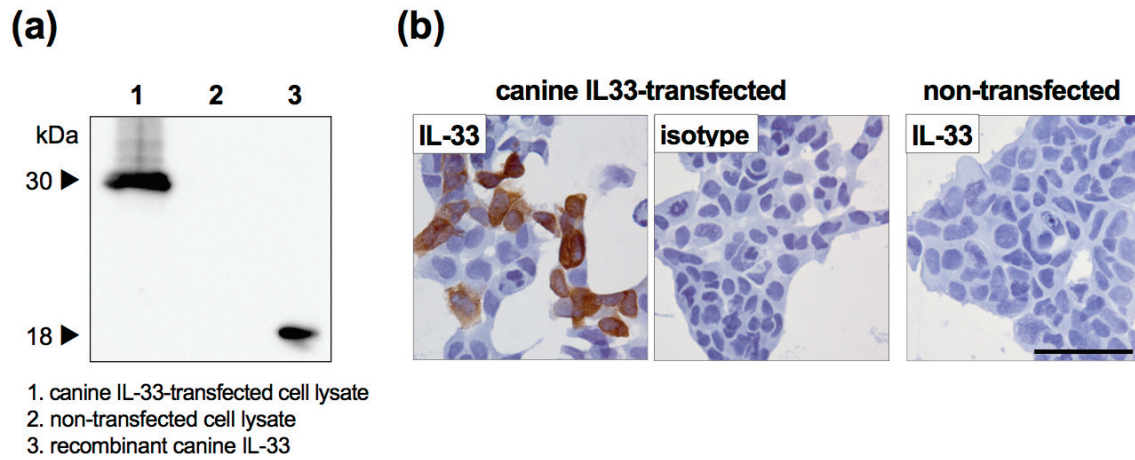


Figure 2-2. (a) Immunoblotting using an anti-human IL-33 antibody in lysates from cells transfected with full-length canine IL-33 and recombinant processed canine IL-33 (expected molecular weights of 30 kDa and 18 kDa, respectively). Non-transfected cells were used as a negative control. (b) Immunocytochemistry in canine IL-33-transfected cells stained with an anti-human IL-33 antibody or isotype control, and in non-transfected cells stained with an anti-human IL-33 antibody. Immunoreactive IL-33 was visualized in light brown, and cells were counterstained with hematoxylin (blue). The scale bar represents 50 μ m.

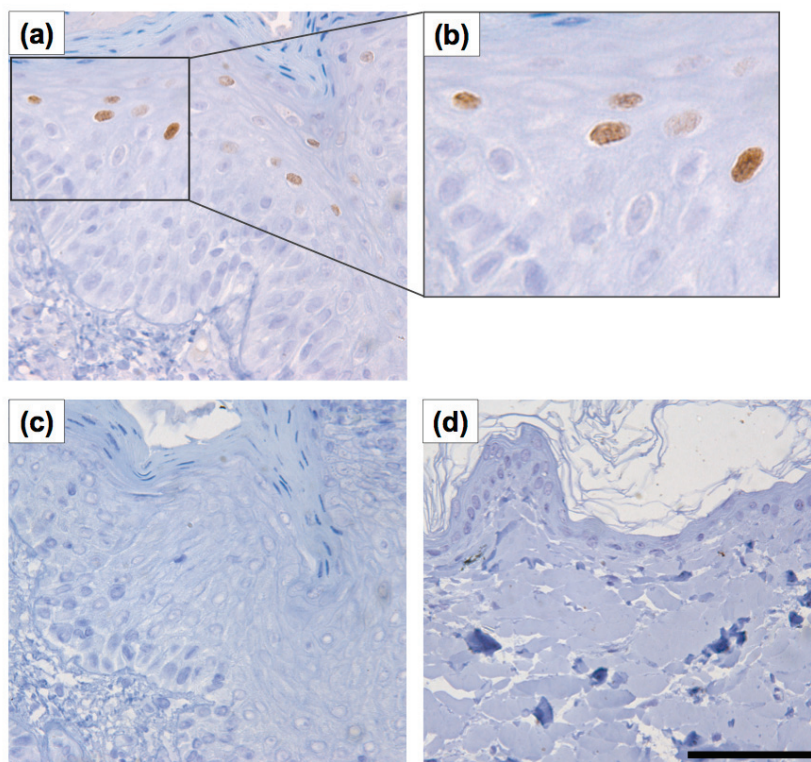


Figure 2-3. Immunohistochemistry in the lesional skin of cAD stained with an anti-human IL-33 antibody (a and b) or isotype control (c), and in normal healthy skin stained with the anti-human IL-33 antibody (d). Immunoreactive IL-33 was visualized in light brown, and sections were counterstained with hematoxylin (blue). The scale bar represents 50 μm in (a), (c), and (d) and 100 μm in (b).

Chapter 3

Transcription of IL-33 induced by IFN-gamma or IL-17A in cultured canine keratinocytes

3. 1. Introduction

In humans and mice, IL-33 binds to its receptor (ST2) [51], which is preferentially expressed on Th2 cells [25,29]. IL-33 was found to enhance the production of IL-5 and IL-13, suggesting that IL-33 has the ability to aggravate Th2-associated inflammation via Th2 cells. Previous studies demonstrated that the expression of IL-33 was up-regulated in the lesional skin of human AD, which is a common Th2-associated inflammatory disease [38,48]. Moreover, serum IL-33 levels were higher in patients with AD than in healthy controls and correlated with severity scores [46,57]. In previous studies using a mouse model of experimental AD, the knockout of IL-33 or a treatment with an anti-IL-33 antibody resulted in improvements in AD-like skin lesions [27,46]. Based on these findings, it is considered that IL-33 is critical for enhancing Th2-associated inflammation in the pathogenesis of human AD. In dogs, accumulating evidence indicates that cAD is generally a Th2-associated inflammatory disease [16,31,32,43,50]. The transcription level of *il-33* was found to be increased in the skin of sensitized dogs after exposure to HDM, a major environmental allergen for cAD [44,49]. The results of chapter 1 revealed that canine Th2 cells preferentially transcribed *st2* and enhanced the transcription of *il-5* and *il-13* after being stimulated with IL-33. The results of chapter 2 showed that the transcription level of *il-33* in the lesional skin of cAD was higher than that in the normal skin of dogs. Therefore, IL-33 appears to play a role in the pathogenesis of cAD as well as human

AD.

In this chapter 2, keratinocytes were identified as the major cellular source of IL-33 in the lesional skin of cAD, as reported in human AD [38,48]. Previous studies using cultured human keratinocytes demonstrated that the expression of IL-33 was induced by IFN-gamma and IL-17A [8,37,38,59], which are up-regulated in the lesional skin of human AD [10,58]. Thus, the expression of IL-33 in keratinocytes was most likely regulated by IFN-gamma and IL-17A in human AD. In canine keratinocytes, the transcription level of IFN-gamma was found to be higher in the lesional skin of cAD than those in the non-lesional and normal skin [30,43,50]. Canine keratinocytes stimulated with IFN-gamma were shown to induce the transcription of *ccl28* [52], suggesting that canine keratinocytes express the receptor for IFN-gamma. IL-17A can induce various soluble factors including GM-CSF, S100 calcium-binding protein A8 (S100A8), IL-8, and IL-19 in canine keratinocytes [1], whereas a direct relationship with IL-17A has not yet been demonstrated in cAD. Consequently, I speculate that the expression of IL-33 in keratinocytes is induced by IFN-gamma and IL-17A in the lesional skin of cAD. However, the effects of IFN-gamma or IL-17A on IL-33 expression in canine keratinocytes have not yet been examined.

In the present study, I investigated whether IFN-gamma or IL-17A induces the transcription of *il-33* in a canine keratinocyte cell line.

3. 2. Materials and Methods

3. 2. 1. Cell culture and stimulation

The canine epidermal keratinocyte progenitor cell line, CPEK, and culture medium (CnT-09) were purchased from CELLnTEC Advanced Cell Systems (Bern, Switzerland). Seventh- to tenth-passaged cells were used in the experiments. Cells were initially cultivated in 75-cm² flasks (Sarstedt, Nümbrecht, Germany) in CnT-09 at 37°C in an atmosphere of air containing 5% CO₂ until approximately 80% confluence was reached. Cultured cells were washed with Dulbecco's phosphate-buffered saline (DPBS; Kohjin Bio Co., Ltd., Saitama, Japan), trypsinized by the addition of 3 mL of recombinant trypsin (TrypLE Express; Invitrogen Corp., Carlsbad, CA, USA), and incubated at 37°C for 5-10 min. When approximately 90% of the cells had been detached, the cell suspension was transferred to a sterile 15-mL centrifuge tube with DPBS and centrifuged at 180×g at room temperature for 5 min. The cell pellet was gently resuspended in CnT-09 and transferred into 24-well plates (Nunc Inc., Roskilde, Denmark) at a cell density of approximately $8 \times 10^4/\text{well}$ ($4 \times 10^4/\text{cm}^2$). When cells had reached 80% confluence in CnT-09, they were washed with DPBS and cultured in serum-free medium (Opti-MEM I; Invitrogen Corp.) for 24 h before a stimulation with rcIFN-gamma or rcIL-17A (R&D Systems, Minneapolis, MN, USA). In order to select the optimal culture duration (0, 6, 12, or 24 h), I initially examined the kinetics of the

transcription of *il-33* in CPEK cells stimulated with rcIFN-gamma or rcIL-17A (10 ng/mL). Cytokine concentrations were based on previous studies [1,52]. Once the optimal culture duration was confirmed to be 24 h, I then examined whether the transcription level of *il-33* may be altered by various concentrations of rcIFN-gamma or rcIL-17A (0, 1, 10, 100, or 500 ng/mL). The concentrations of rcIFN-gamma and rcIL-17A employed in the present study were not shown to have cytotoxic affect in previous reports [1,52].

3. 2. 2. RT-qPCR

The steps from total RNA extraction to RT-PCR were carried out as described in this chapter 1. The primer pairs used in this study are listed in Table 3-1. The specificities of these primers were confirmed to amplify each target mRNA by a sequential analysis of PCR products using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). RT-qPCR was performed using SYBR Premix Ex TaqTM II (Takara Bio Inc.). In order to ensure accurate quantification, two reference genes including RPL32 and RPS18 were selected by GeNorm (<https://genorm.cmgg.be>) among five candidate reference genes (HPRT1, GAPDH, RPL13A, RPL32, and RPS18) [47]. Amplification consisted of a first period of activation (95°C for 10 s) followed by 40 cycles of a PCR reaction (95°C for 5 s and 60°C for 30 s) and dissociation (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s). Each

PCR reaction included samples without reverse transcription, which were not amplified in the present study. Ct values, which indicate the point at which the threshold intersects with the amplification curve of the PCR reaction, were obtained using software (Thermal Cycler Dice Real Time System Multiplate RQ software, Version 3.00; Takara Bio Inc.). Each transcription level (relative quantity) was quantified using the comparative Ct ($2^{-\Delta\Delta Ct}$) method, by which the transcription level of the target gene in each sample was represented as an n -fold difference from that in the calibrator sample. The Ct values of the target genes were subtracted from the geometric mean of the Ct values of the two reference genes (ΔCt) [62]. All samples were examined in duplicate, and the mean value of ΔCt was calculated. The relative quantity of target genes was calculated by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{samples}} - \Delta Ct_{\text{calibrator}}$, using unstimulated cells as the calibrator sample. The mean relative quantity was described by the ratio of stimulated samples to unstimulated samples with the SD of three independent experiments.

3. 2. 3. *Statistical analysis*

Dunn's tests were used for comparisons of the transcription level of each gene among different culture periods or concentrations. A value of $P < 0.05$ was considered to be significant. Statistical analyses were performed using the JMP 10.0 program (SAS Institute, Cary, NC, USA).

3. 3. Results

The transcription levels of *il-33* significantly increased and peaked 24 h after the stimulation with rcIFN-gamma or rcIL-17A (Figure 3-1). The patterns of the transcription level of *il-33* were explained by a bell-shaped concentration-response curve, the maximum effect of which was observed at 100 ng/mL (Figure 3-2).

3. 4. Discussion

In this chapter, I demonstrated that the transcription of *il-33* was up-regulated by IFN-gamma and IL-17A in canine keratinocytes. These results are similar to previous findings in human keratinocytes [8,37,38,59]. Previous studies using human keratinocytes also found that IFN-gamma or IL-17A induced the expression of full-length IL-33, which was detected in the cell lysates, but not culture supernatant of stimulated keratinocytes [8,37,38,59]. The expression of full-length IL-33 as a nuclear protein [5] suggests its release from damaged keratinocytes [57]. The combined treatment with IFN-gamma and TNF-alpha induced the expression of processed IL-33, which was the cleaved N terminal domain of full-length IL-33 by calpain, an intracellular Ca^{2+} -dependent cysteine protease [38]. A previous study demonstrated that processed IL-33 cleaved by calpain was secreted from human gastric carcinoma cells [14]. Thus, human keratinocytes stimulated with IFN-gamma may secrete processed IL-33 in cooperation with TNF-alpha. These findings prompted us to investigate whether processed IL-33 was present in the culture supernatant of canine keratinocytes co-stimulated with IFN-gamma and TNF-alpha. To evaluate the processed IL-33, immunoblotting should be conducted in future studies.

In human AD, chronic lesions show the increased expression of IFN-gamma [10]. IFN-gamma is known to induce the production of cytokines and chemokines, such as IL-1, IL-6, IL-18, CCL17, CCL22, CCL28, and CXC motif chemokine ligand

10 (CXCL10), in human keratinocytes [63], indicating that IFN-gamma contributes to chronic inflammation in human AD. In dogs, the transcription level of *ifn-gamma* in chronic lesions of cAD was higher than that in the non-lesional and normal skin of dogs [30,43,50]. Additionally, a transcriptional analysis of the lesional skin of cAD revealed an increased transcription level of *ccl28* [33]. These findings implicate IFN-gamma in the pathogenesis of cAD through the activation of keratinocytes. In the results of this chapter, IFN-gamma is found to induce the transcription of *il-33* in canine keratinocytes. A previous study revealed the presence of IFN-gamma-producing CD4⁺ cells, which are classified as Th1 cells, in the lesional skin of cAD [19]. Therefore, IFN-gamma produced by Th1 cells may act as an inducer of IL-33 in the lesional skin of cAD.

IL-17A is a cytokine that is mainly secreted by Th17 cells. Previous studies demonstrated that the number of Th17 cells was increased in acute lesions of human AD [21,60]. The IL-17A receptor on keratinocytes was also shown to be expressed in the lesional skin of human AD [42]. The stimulation of IL-17A *in vitro* increased the expression of GM-CSF, IL-8, IL-19, and S100A8 in human keratinocytes [21,40,61]. Based on these findings, it is considered that keratinocytes activated by IL-17A are the most likely to be involved in the pathogenesis of human AD. A direct relationship with IL-17A has not yet been demonstrated in cAD; however, canine keratinocytes stimulated with IL-17A have the ability to induce the transcription of *s100a8* [1], which is an antimicrobial peptide that is up-regulated in the lesional skin of cAD [64].

My results of this chapter demonstrated that IL-17A induced the transcription of *il-33* in canine keratinocytes. Thus, I speculate that IL-17A produced by Th17 cells acts as the inducing factor for the production of IL-33 in the lesional skin of cAD. In order to prove this hypothesis, additional studies that investigate whether IL-17A contributes to the pathogenesis of cAD are needed.

In conclusion, the present results provide evidence to show that IFN-gamma and IL-17A have the ability to induce the transcription of *il-33* in canine keratinocytes. Further studies are needed in order to elucidate whether IFN-gamma and IL-17A play an important role for producing IL-33 in the pathogenesis of cAD.

3. 5. Table and Figure

Table 3-1. Sequences of primers for RT-qPCR including cytokine and reference genes.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
IL-33	GTACTTTATGCAACTGCGTTCTGG	CAGACATTGCTTTCTGCACTTTTC
RPL32	TGGTTACAGGAGCAACAAGAAA	GCACATCAGCAGCACTTCA
RPS18	TGCTCATGTGGTATTGAGGAA	TCTTATACTGGCGTGGATTCTG

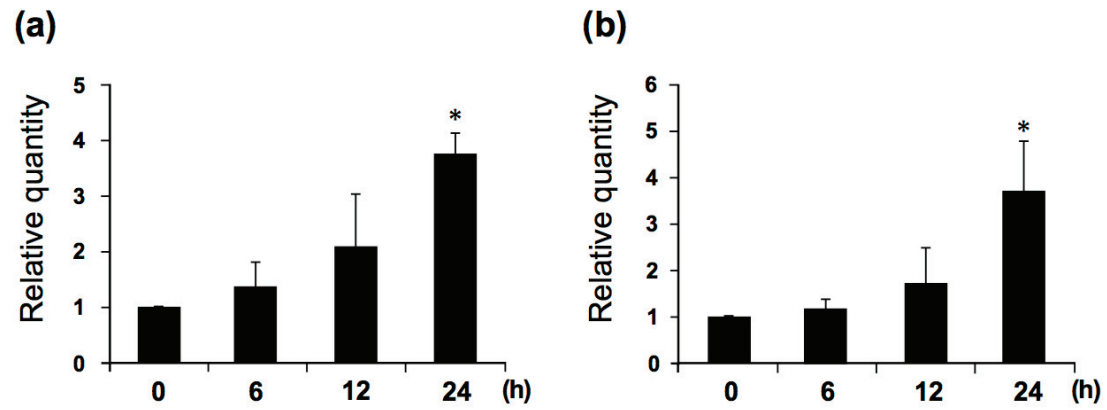


Figure 3-1. Kinetics of the transcription of *il-33* in CPEK cells stimulated with rcIFN-gamma (a) and rcIL-17A (b) at 10 ng/mL. The relative quantity of *il-33* in stimulated cells represents an *n*-fold difference from that in unstimulated cells. Results represent the means \pm SD of three independent experiments. * $P < 0.05$.

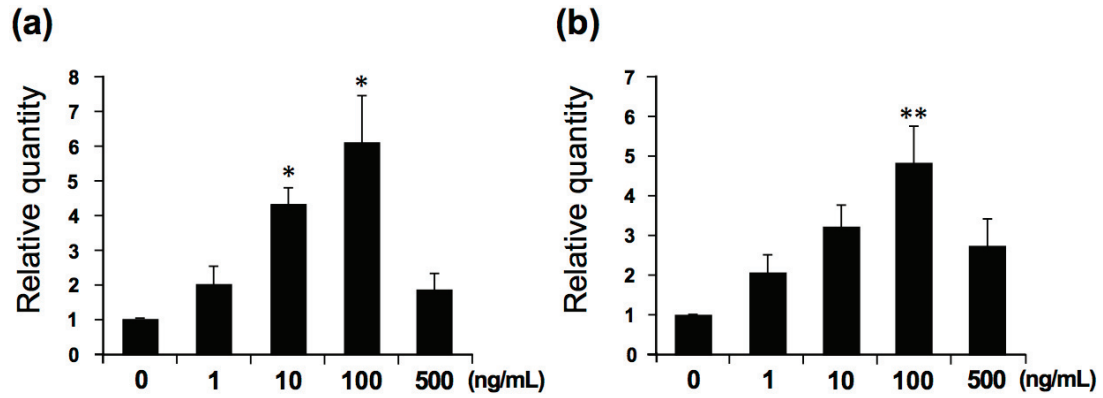


Figure 3-2. Dose-dependent studies on the effects of rcIFN-gamma (a) and rcIL-17A (b) on the transcription of *il-33* in CPEK cells. The relative quantity of *il-33* in stimulated cells represents an n -fold difference from that in unstimulated cells. Results represent the means \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

General Conclusion

Canine AD is a common skin diseases characterized by Th2-associated inflammation, similar to human AD [16,31,32,43,50]. Several studies have shown that IL-33, a novel keratinocyte-derived cytokines, contributes to the enhancement of Th2-associated inflammation via Th2 cells in the pathogenesis of human AD [18,48]. Serum IL-33 levels can be useful as biomarker of human AD [18,57]. Furthermore, the potential benefits of an anti-IL-33 monoclonal antibody have recently been demonstrated in a mouse model of experimental AD [46]. These findings indicate that IL-33 is a novel molecular target for biomarker and therapeutic strategy for human AD; however, the investigation focused on IL-33 in cAD has never been investigated. Therefore, I carried out a series of studies to clarify the involvement and pathophysiological roles of IL-33 in cAD.

In chapter 1, I performed transcriptional analysis of *st2* in T cell subsets and *th2 cytokines* in canine Th2 cells stimulated with IL-33. I found that canine Th2 cells preferentially expressed ST2 and transcribed *il-5* and *il-13* genes after being co-stimulated with IL-33 and IL-2. These results indicate that IL-33 has the ability to enhance Th2-associated inflammation via Th2 cells in cooperation with IL-2.

In chapter 2, I performed transcriptional and immunohistochemical analysis of IL-33 in the lesional skin of cAD. I found that the lesional skin had higher transcription level of IL-33 than the normal skin of dogs. I also found that keratinocytes were a major cellular source of IL-33 production in the lesional skin of cAD. These findings indicated that keratinocyte-derived IL-33 is implicated in the

immunopathogenesis of cAD.

In chapter 3, I carried out transcriptional analysis of *il-33* in cultured canine keratinocytes stimulated with IFN-gamma or IL-17A. I found that the transcription of *il-33* in cultured keratinocytes was enhanced after stimulation with IFN-gamma or IL-17A. My results suggest that these cytokines, which are mainly produced from Th1 and Th17 cells, act as important inducing factors for the production of IL-33 in the lesional skin of cAD.

In conclusion, this is the first study to elucidate the biological immune function of keratinocytes that enhance cytokine production of Th2 cells by producing IL-33 in dogs. The results obtained in a series of my doctoral study indicated that IL-33 may be a key keratinocyte-derived cytokine for enhancing allergic inflammation via Th2 cells in the lesional skin of cAD, which provide new paradigms for understanding its immunopathogenesis. Prospective studies should be conducted to investigate whether IL-33 can be used as a novel biomarker or therapeutic strategy for cAD.

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