



Interference of Skin Scratching Attenuates Accumulation of Neutrophils in Murine Allergic Contact Dermatitis Model

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Abstract—We recently reported that swelling resulting from 2,4,6-trinitrochlorobenzene (TNCB) challenge might be associated with recruitment of neutrophils. However, it is not known whether neutrophil recruitment is affected by scratching at inflamed sites or not. Therefore, the effects of an Elizabethan collar on the TNCB-induced upregulation of ELR-positive chemokines (CXCL1, CXCL2, and CXCL5) and neutrophil recruitment were investigated. Mice were sensitized by the application of TNCB on abdominal skin. Then, the mice were challenged three times with TNCB to auricle of the ear. To prevent scratching at inflamed sites, an Elizabethan collar was placed on the mice from just before the first challenge until the end of the experiment. The effects of the Elizabethan collar on the TNCB-induced upregulation of CXCLs chemokines and recruitment of neutrophil were investigated. The increase of ear swelling by TNCB challenge was inhibited by the Elizabethan collar. TNCB-challenge-induced upregulation of TNF- α , IL-1 β , IL-6, ELR⁺ chemokines, MPO, and ELA2 was also attenuated by the Elizabethan collar. The gene expression of CXCL1, CXCL2, and CXCL5 human homolog IL-8 was enhanced by TNF- α and IL-1 β in human dermal fibroblasts and epidermal keratinocytes. We here suggest that scratching the site of inflammation leads to neutrophil accumulation mediated by TNF- α and IL-1 β /ELR⁺ chemokines in TNCB-challenge-induced contact dermatitis in mice.

KEY WORDS: allergic contact dermatitis; itch sensation; skin scratching; chemokine; neutrophil.

INTRODUCTION

Allergic contact dermatitis (ACD) develops after someone encounters an allergen or hapten, which is a small molecule. ACD potentially leads to itching, swelling, redness, and rash. Common causes of ACD include perfume, eye shadow, nail polish, lipstick, some sunscreens, and dyes in clothing. CD is, thus, a frequent occupational skin disease and one of the major environmental health problems in developed countries [4, 12]. CD is classified as a

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type IV allergy [10, 19]. It may start to develop more than 24 h after exposure. It involves a type IV hypersensitivity or delayed reaction performed by T cells. It is widely appreciated that skin inflammation can lead to itch [2]. Although the precise mechanisms by which pro-inflammatory mediators promote the sensation of pruritus remain poorly defined, the type 2 cytokines IL (interleukin)-4 and IL-13 are known to drive skin inflammation in allergic dermatitis [8, 34]. The sensation of itch, which induces scratching in order to relieve it, is uncomfortable. Scratching can also often intensify itching and cause further damage to the skin [17, 25]. However, the detailed mechanisms behind the influence of factors induced by scratching in inflamed skin are unclear.

Neutrophils are the first immune cells that are recruited to the site of inflammation and have mainly been considered in the context of acute inflammation during hypersensitivity. CXC chemokine receptor 2 (CXCR2) is a receptor for CXC chemokines bearing the glutamic acid–leucine–arginine motif (ELR-positive chemokines). ELR-positive chemokines are mainly responsible for neutrophil chemotaxis and contribute to neutrophil migration, and seven ELR⁺ CXC chemokines have been identified in humans: CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and IL-8 [16]. Among these human chemokines, IL-8 is the most potent neutrophil chemoattractant, and its analogs in mice are CXCL1, CXCL2, and CXCL5, which act through the receptor CXCR2 and appear to confer selectivity for promoting neutrophil chemotaxis [5, 11, 14–16].

Recently, we reported that the mRNA and protein levels of ELR⁺ chemokines CXCL1, CXCL2, and CXCL5 were markedly increased at inflamed sites in TNCB-induced ACD in mice. In addition, increases in myeloperoxidase (MPO) activity induced by TNCB challenge were observed in contact dermatitis-affected regions. These findings show that swelling resulting from TNCB challenge might be associated with upregulated ELR-positive chemokine (CXCL1, CXCL2, and CXCL5)-induced recruitment of neutrophils. On the other hand, it has been known that tape stripping, a surrogate for scratching, causes neutrophil accumulation in mouse skin [24]. However, it is not known whether ELR-positive chemokines are affected by actual scratching at inflamed sites. Toenail clipping is a classical method to interfere with the scratching effect [30]. Even with nail clippers, it is impossible to eliminate the stimulation of the diseased part. Therefore, we used the Elizabethan collar, which can almost completely inhibit ear scratching. In the present study, to investigate whether ELR-positive chemokines are affected by scratching at inflamed sites, mice were equipped

with an Elizabethan collar to prevent them from scratching inflamed sites. The effects of the Elizabethan collar on the TNCB-induced upregulation of ELR-positive chemokines and recruitment of neutrophils were then investigated.

MATERIALS AND METHODS

Mice

Female BALB/c mice (7–8 weeks old) were purchased from the Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan) and housed in a pathogen-free facility.

Model of Allergic Contact Dermatitis and Use of Elizabethan Collar

Mice were sensitized as described previously [27]. Sensitized control (S.C.) mice were treated with 100 μ L of 5.0% (w/v) TNCB at day 0 and acetone alone at days 5, 8, and 11. The determination of ear thickness (along with various other analyses) was performed 24 h after the final challenge with a dial thickness gauge (G-7C; Ozaki, Tokyo, Japan). The Elizabethan collar (Natsume Seisakusho Co., Ltd., Tokyo, Japan) was applied from just before the first challenge until the end of the experiment.

Histology

Standard hematoxylin and eosin (HE) staining was performed on each ear. Briefly, the ear was removed from anesthetized mice, fixed with 10% formalin, paraffin embedded, sectioned, and HE stained.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

The mRNA expression was measured by qRT-PCR. Briefly, total RNA was extracted from murine auricle with a one-step guanidium–phenol–chloroform extraction procedure using TRI ReagentTM (Sigma-Aldrich, St. Louis, MO, USA). cDNA was prepared from total RNA (1.0 μ g) with QuantiTect Reverse Transcriptase (Qiagen, Hilden, Germany) after incubation with gDNA wipeout buffer at 42 °C for 2 min to remove contaminating genomic DNA. Reaction mixtures (3 μ L) were subjected to PCR (50 nM forward and reverse primers, Fast SYBR Green Mastermix; Applied Biosystems, DriveFoster, CA, USA) in a final volume of 10 μ L. The thermal cycle profiles used were (1) denaturing for 30 s at 95 °C and (2) annealing for 30 s at 60 °C. PCR amplification was performed for 40 cycles. Data are presented as the mRNA expression

relative to that of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta C_t}$ method. The PCR primer sets used are shown in Table 1.

Immunoblotting

Immunoblotting was performed as described previously [18]. Briefly, to prepare homogenates, ear tissues were removed and immediately soaked in ice-cold phosphate-buffered saline. Tissue was then homogenized in ice-cold T-PER™ Tissue Protein Extraction Reagent (Life Technologies, Carlsbad, CA, USA). The tissue homogenate was centrifuged ($1000\times g$, 4 °C for 15 min) and supernatants stored at -80 °C until use. Samples (10 μ g total protein per lane) were separated by 5–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then electrophoretically transferred to a PVDF (polyvinylidene difluoride) membrane. After blocking with 3% bovine serum albumin, the transferred PVDF membranes were incubated with the primary antibodies. The primary antibodies used were rabbit anti-CXCL1 (1:1000 dilution; Abcam, Cambridge, UK), mouse CXCL2 (1:1000; PeproTech, Rocky Hill, NJ, USA), mouse CXCL5 (1:1000; PeproTech), mouse

myeloperoxidase (1:1000; R&D Systems, Minneapolis, MN, USA), mouse neutrophil elastase (ELA2) (1:1000; R&D Systems), and rabbit GAPDH (1:5000; Cell Signaling Technology Japan, K.K., Tokyo, Japan). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, and bands detected with an ECL system (Immuno Star LD, Wako Pure Chemical Industries Ltd., Osaka, Japan), following manufacturer's instructions. The secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG; 1:5000 dilution; Cell Signaling Technology Japan) and anti-mouse immunoglobulin G (IgG; 1:5000 dilution; Cell Signaling Technology Japan).

In vivo Imaging for Myeloperoxidase (MPO) Activity

Twenty-four hours after the final TNCB challenge, each mouse was intraperitoneally injected with a Xenolight RediJect Inflammation Probe (0.2 g/kg body weight; Perkin Elmer, Waltham, MA, USA). Then, *in vivo* bioluminescence in the anesthetized mice was captured by IVIS® Lumina (Perkin Elmer) 10 min after injection, with a 5-min exposure time. The images were quantified by Living Image Software (Perkin Elmer).

Table 1. PCR Primers Used in the Present Study

Species	Accession number	Primers	Deoxyribonucleotide sequences		Product size (base pairs)
			forward	reverse	
<i>Mus musculus</i>	GAPDH	NM_008084.2	forward	CCTCGTCCCGTAGACAAAATG	100
			reverse	TCTCCACTTTGCCACTGCAA	
<i>Mus musculus</i>	TNF- α	NM_013693.2	forward	GGCAGGTTCTGTCCCTTCA	115
			reverse	GGAGTGCCTCTTCTGCCAGTT	
<i>Mus musculus</i>	IL-1 β	NM_008361.3	forward	TCGTGCTGTCGGACCCATAT	111
			reverse	TGTCGTTGCTTGTTCTCCTT	
<i>Mus musculus</i>	IL-6	NM_031168.1	forward	GACTTCCATCCAGTTGCCTTCT	115
			reverse	AGACAGGTCTGTTGGGAGTGGTA	
<i>Mus musculus</i>	IL-4	NM_021283.2	forward	CATATCCACGGATGCGACAA	112
			reverse	CTGTGAGGACGTTTGGCACAT	
<i>Mus musculus</i>	IL-13	NM_008355.3	forward	CTGGTCCACACAGGGCAACT	110
			reverse	CCCACCGGATACTGACAGA	
<i>Mus musculus</i>	IFN- γ	NM_008337.3	forward	AGCAACAGCAAGGCGAAAAA	110
			reverse	TGGTGGACCACTCGGATGA	
<i>Mus musculus</i>	CXCL1	NM_008176.3	forward	GCTCCCTTGGTTCAGAAAATTG	97
			reverse	TCACCAGACAGGTGCCATCA	
<i>Mus musculus</i>	CXCL2	NM_009140.2	forward	CCTGCCAAGGGTTGACTTCA	105
			reverse	TTTGACCGCCCTGAGAGT	
<i>Mus musculus</i>	CXCL5	NM_009141.3	forward	GTGGAAAGAACGGCCAGTGT	96
			reverse	AATCCGTGGGTGGAGAGAATC	
<i>Homo sapiens</i>	GAPDH	NM_002046.7	forward	ACAACCTTGGTATCGTGGAAAG	101
			reverse	GCCATCACGCCACAGTTTC	
<i>Homo sapiens</i>	IL-8	NM_000584.4	forward	ACTGAGAGTGATTGAGAGTGGAC	112
			reverse	AACCCTCTGCACCCAGTTTC	

Cell Culture

Normal human dermal fibroblasts (-Neonatal) were grown under standard conditions [MEM- α , with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml)] in an incubator containing 5% CO₂ at 37 °C and were treated with 10 or 30 ng/mL TNF- α , IL-1 β , and IL-6 for 24 h. HaCaT human keratinocytes were incubated in Ca²⁺-free DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml) in an incubator containing 5% CO₂ at 37 °C and were treated with 10 or 30 ng/mL TNF- α , IL-1 β , and IL-6 for 24 h.

Statistical Analyses

Indicators of statistical significance were generated using GraphPad Prism 5 for Mac OS X (GraphPad Software, Inc., CA, USA). Results are expressed as mean \pm

SEM. Statistical significance of difference was determined by unpaired Student's *t* test or two-way analysis of variance (ANOVA) with *post hoc* Bonferroni for differences among individual groups. A value of *P* < 0.05 was considered significant.

RESULTS

Effect of Elizabethan Collar on 2,4,6-Trinitrochlorobenzene (TNCB)-Challenge-Induced Swelling of the Ear

The mice were equipped with an Elizabethan collar to keep the challenge site scratch-free (Fig. 1a). Histopathology showed an increased thickness of TNCB-challenged ears compared with that in the S.C. group (Fig. 1b-d). We

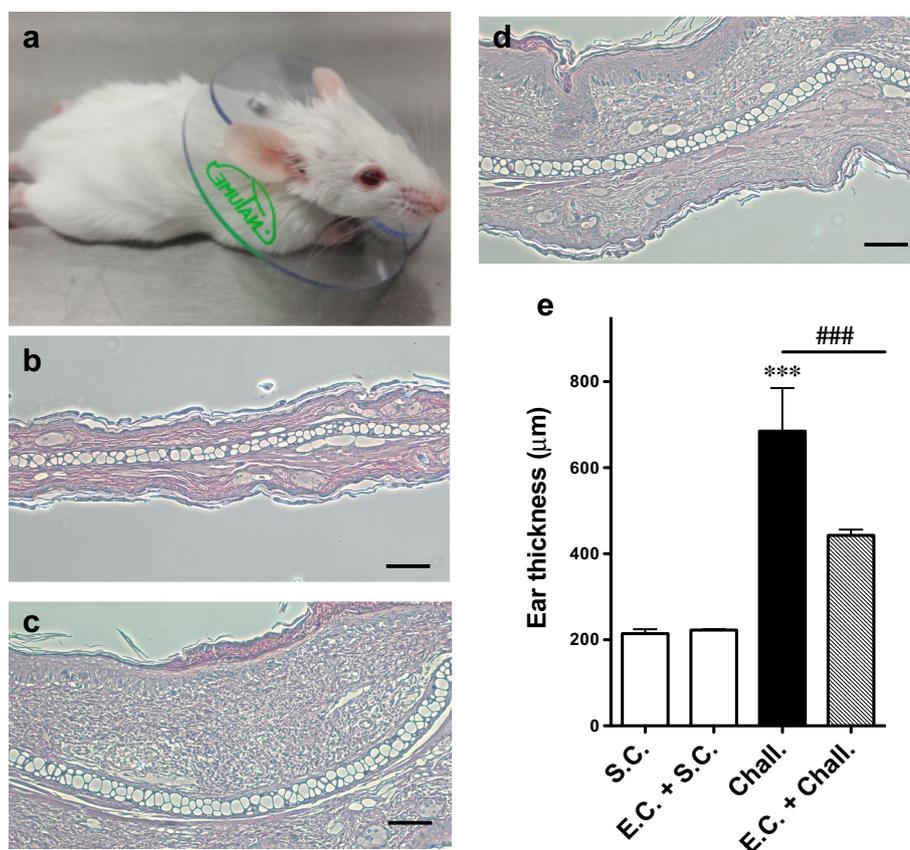


Fig. 1. Effect of Elizabethan collar on the 2,4,6-trinitrochlorobenzene (TNCB)-challenge-induced swelling of the ear. The Elizabethan collar (E.C.) was applied from just before the first challenge until the end of the experiment. **a** Representative photomicrographs of hematoxylin-eosin (HE)-stained auricle 1 day after the last challenge [**b** sensitized control (S.C.); **c** Chall.; **d** E.C. + Chall.]. The TNCB-challenge-induced swelling of the ear was inhibited by the Elizabethan collar (**e**). Each column represents the means with SEM from 4 to 8 experiments. ****P* < 0.001 vs. sensitized control (S.C.). ###*P* < 0.001 vs. TNCB-challenged groups (Chall.). Scale bar = 50 μ m.

examined ear thickness 24 h after the final TNCB challenge and found that it was markedly thickened by TNCB challenge. Interestingly, the increase in ear swelling by

TNCB challenge was inhibited by the Elizabethan collar, although ear thickness was unchanged by the use of the Elizabethan collar in the S.C. group (Fig. 1b-e).

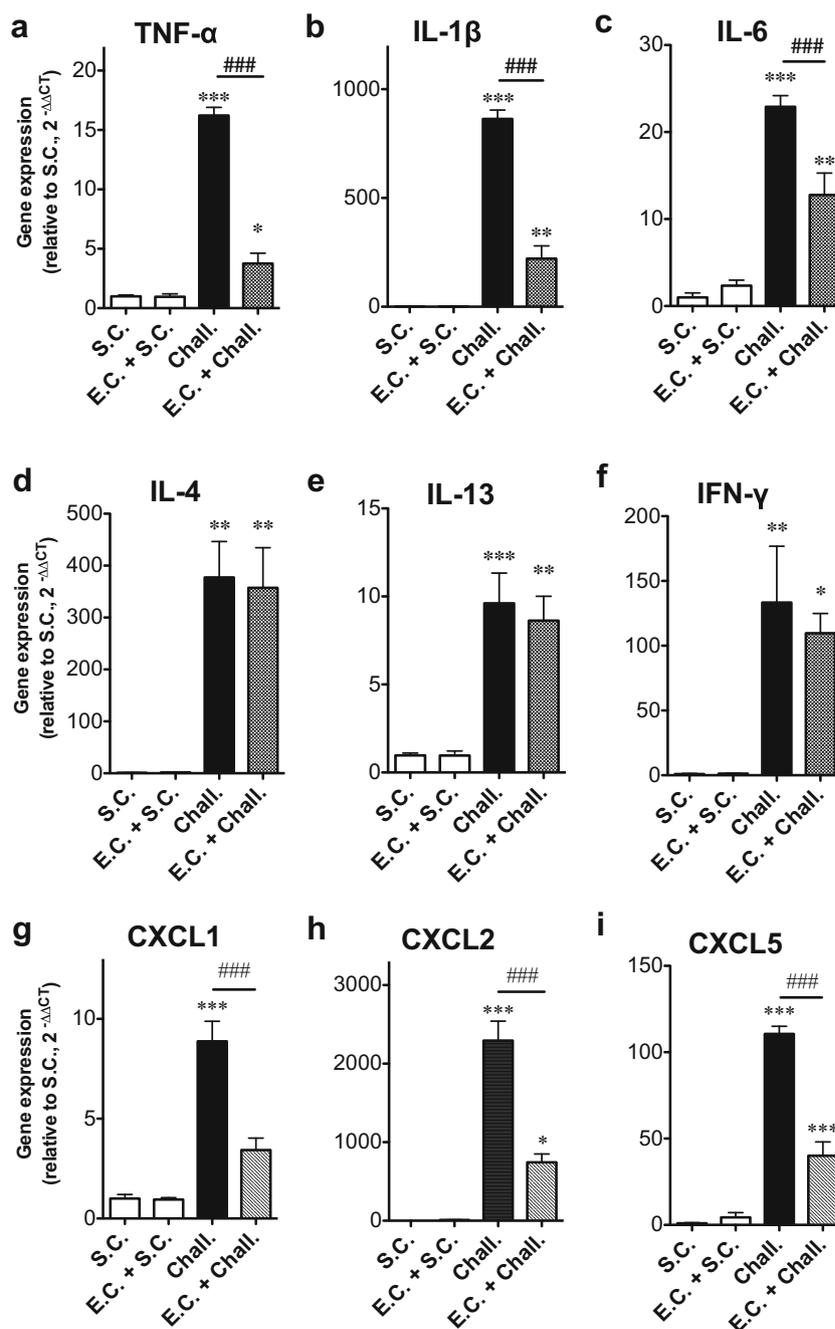


Fig. 2. Effect of Elizabethan collar on the TNCB-challenge-induced upregulation of gene expression of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6), IL-4, IL-13, IFN- γ , and ELR⁺ chemokines (CXCL1, CXCL2, and CXCL5) in the ear. Results are expressed as the mean \pm SEM from four independent experiments. ** P < 0.01 and *** P < 0.001 vs. sensitized control (S.C.). #### P < 0.001 vs. Chall.

There was no difference in weight change of day 12 (24 h after the final TNCB challenge) to day 0 (day of sensitization) in all groups (data not shown).

Effect of Elizabethan Collar on TNCB-Challenge-Induced Upregulation of Cytokine and ELR+ Chemokine Genes in the Ear

The expression of genes encoding inflammatory cytokines TNF- α , IL-1 β , IL-6, IL-4, IL-13, and IFN- γ was significantly enhanced by the TNCB challenge compared with that in the S.C. group. Among them, TNCB-challenge-induced upregulation of TNF- α , IL-1 β , and IL-6 gene expression was attenuated by the Elizabethan collar. The gene expression of ELR⁺ chemokines CXCL1, CXCL2, and CXCL5 was markedly increased by the TNCB challenge compared with that in the S.C. group. The TNCB-challenge-caused upregulation of ELR+

chemokine genes in the ear was decreased by the Elizabethan collar (Fig. 2G-I).

We next investigated the protein levels of CXCL1, CXCL2, and CXCL5 in TNCB-challenged mouse ear. Protein levels of CXCL1, CXCL2, and CXCL5 were also increased in TNCB-challenged ear compared with those in the S.C. group. The TNCB-challenge-induced upregulation of ELR-positive chemokine proteins in the ear was decreased by the Elizabethan collar (Fig. 3a-d).

Effect of Elizabethan Collar on the Expression of Myeloperoxidase (MPO), Neutrophil Elastase (ELA2), and MPO Activity, in TNCB-Challenged Ears

To examine recruitment of neutrophils in the auricle of ear, we measured the protein levels of myeloperoxidase (MPO) and neutrophil elastase (ELA2). Both are major proteins in neutrophil granules and are frequently used as neutrophil markers [9, 26, 31]. The MPO and ELA2

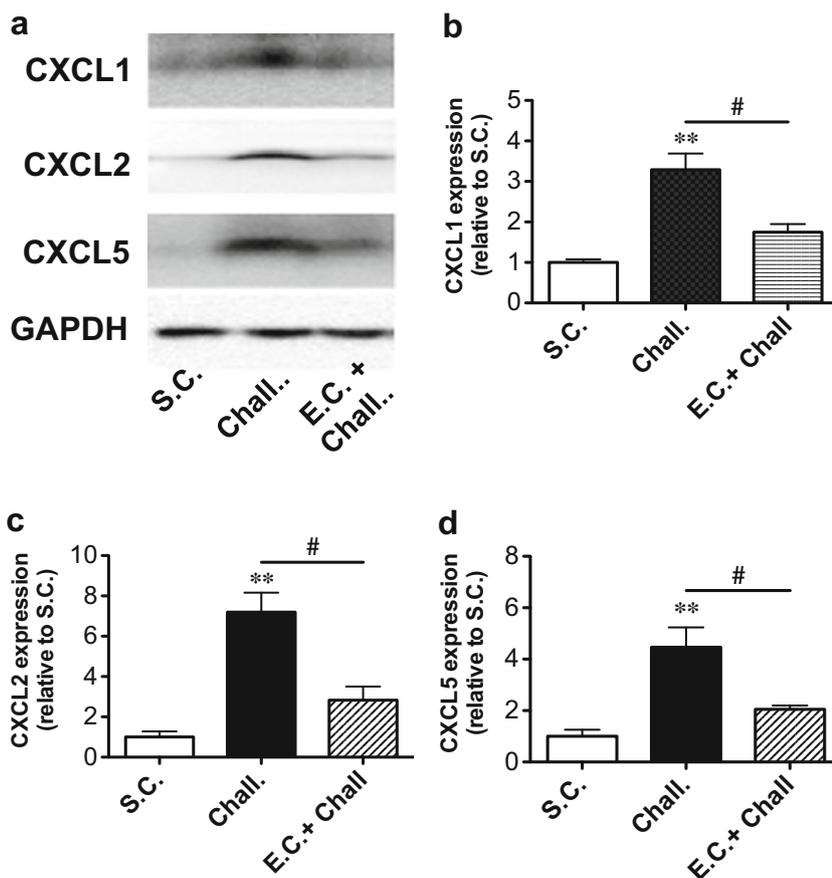


Fig. 3. Effect of Elizabethan collar on the TNCB-challenge-induced upregulation of protein levels of the ELR⁺ chemokines CXCL1, CXCL2, and CXCL5 in mouse ears. Representative photographs showing bands for CXCL1, CXCL2, CXCL5, and the internal control, GAPDH (a). Results are expressed as the mean \pm SEM from four experiments (b-d). ** P < 0.01 vs. sensitized control (S.C.). # P < 0.05 vs. Chall.

expression levels were upregulated by TNCB challenge. The TNCB-induced increases in the levels of MPO and ELA2 protein were inhibited by the use of an Elizabethan collar (Fig. 4a–c). We next measured MPO activity among the groups using a XenoLight RediJect Inflammation Probe and an IVIS Lumina (*in vivo* imaging system). The images of TNCB-challenged ears revealed striking differences in the level of luminescence in TNCB-challenged mice compared with that in S.C. mice. The emission signal was localized and markedly increased in the ears of TNCB-challenged mice compared with that in controls. In addition, TNCB-challenge-induced increases in MPO activity were markedly attenuated by the Elizabethan collar (Fig. 4d).

Pro-Inflammatory Cytokine TNF- α , IL-1 β , and IL-6-Induced Changes in IL-8 Gene Expression in Human Dermal Fibroblasts and Epidermal Keratinocytes

Finally, we examined whether TNF- α , IL-1 β , and IL-6 induced the gene expression of CXCL1, CXCL2, and CXCL5 homolog human IL-8 in human dermal fibroblasts and epidermal keratinocytes. IL-8 expression was enhanced by 10–30 ng/mL TNF- α and IL-1 β , but not changed by IL-6 treatment in human dermal fibroblasts (Fig. 5A) and epidermal keratinocytes (Fig. 5B). The increase in expression of the IL-8 gene by TNF- α and IL-1 β was more pronounced in fibroblasts than in keratinocytes.

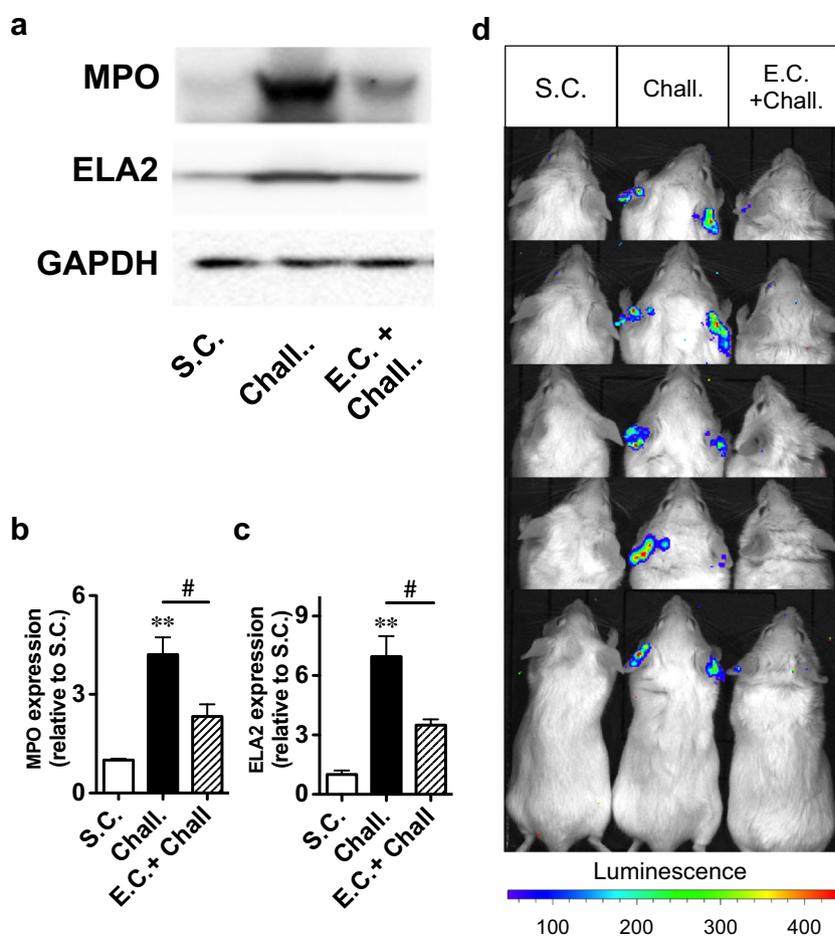


Fig. 4. Effect of Elizabethan collar on the expression of myeloperoxidase (MPO), neutrophil elastase (ELA2), and MPO activity, in TNCB-challenged ears. Representative photos showing bands for ELA2, MPO, and the internal control, GAPDH (a). Levels of ELA2 and MPO expressed as the ratios of the intensities of MPO and ELA2 relative to GAPDH protein bands (b, c, respectively). Results are expressed as the mean \pm SEM from four experiments. ** $P < 0.01$ vs. sensitized control (S.C.). # $P < 0.05$ vs. Chall. MPO activity among sensitized controls (S.C.), saline + challenged ears (Chall.), and Elizabethan collar (E.C) + challenged ears (E.C. + Chall.) (d). *In vivo* imaging photographs of luminescence level are presented from five independent experiments.

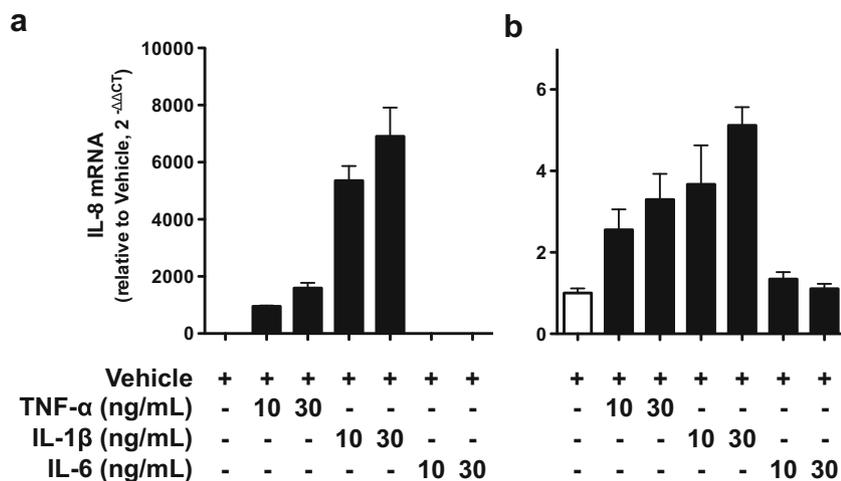


Fig. 5. Pro-inflammatory cytokines induced a change of IL-8 gene expression in human dermal fibroblasts and epidermal keratinocytes. The gene expression of IL-8 was increased with 10 or 30 ng/mL TNF- α and IL-1 β , but not with IL-6 in the human dermal fibroblasts (A) and epidermal keratinocytes (B). Results are expressed as the mean \pm SEM from three independent experiments.

DISCUSSION

In dermatitis patients, histamine H₁ receptor antagonists are the first-choice drugs for pruritus therapy; however, they often fail to reduce the pruritus in dermatitis patients [13, 22, 33]. Therefore, the pruritus in patients with atopic dermatitis may have diverse causes and involve not only histamine but also unknown pruritogens, such as opioid receptor agonists, substance P, and 5-hydroxytryptamine [1, 3, 20, 36]. Therefore, the detailed mechanisms behind the development of pruritus in dermatitis patients remain unclear. If itching and scratching behavior can be stopped, it was assumed that this could limit the symptoms of dermatitis. In the present study, we blocked scratching using an Elizabethan collar in the TNCB-induced contact dermatitis mouse model. We here present that the TNCB-induced upregulation of the pro-inflammatory cytokines TNF- α ; IL-1 β ; IL-6; ELR-positive chemokines CXCL1, CXCL2, and CXCL5; MPO; and ELA2 was attenuated by the use of an Elizabethan collar. TNCB-induced swelling was also inhibited by this collar. Recently, we reported that the ELR-positive chemokines play crucial roles in contact hypersensitivity. The report also indicated that the ear swelling induced by TNCB challenge was inhibited by SB225002, a CXCR2 antagonist, and was associated with a reduction in recruitment of neutrophil [28]. Taking these findings together, TNCB-challenge-induced recruitment of neutrophils in the ear auricle might be exacerbated by scratching of the inflamed site.

It is well known that CXCL1, CXCL2, and CXCL5 can be induced by pro-inflammatory cytokines, such as TNF- α and IL-1 β , in various cells [6, 7, 21, 32, 35, 37]. In the current study, we suggested that the TNCB-challenge-induced upregulation of TNF- α and IL-1 β gene expression was attenuated by the use of an Elizabethan collar and that the gene expression of the CXCL1, CXCL2, and CXCL5 homolog human IL-8 was increased by treatment with TNF- α and IL-1 β , but not with IL-6. Taking these findings together, it is suggested that the increased expression of CXCL1, CXCL2, and CXCL5 in TNCB-challenge-induced contact dermatitis is partly mediated by the increased expression of TNF- α and IL-1 β .

Pruritus is one of the most irritating symptoms characterizing dermatitis and impairs the quality of life. Although the pathogenesis of pruritus remains unclear, recent studies have shown the presence of sensory nerve fiber hyperinnervation of the epidermis in patients with atopic dermatitis [23]. Furthermore, itching can be caused not only by changes to the peripheral nervous system but also by those to the central nervous system. Shiratori-Hayashi et al. [29] demonstrated that signal transducer and activator of transcription 3-dependent reactive astrocytes act as critical amplifiers of pruritus sensation mediated by the enhancement of spinal itch signals by lipocalin-2.

In conclusion, we here suggest that scratching the site of inflammation leads to neutrophil accumulation, which is mediated by TNF- α and IL-1 β /CXCLs, in TNCB-challenge-induced contact dermatitis in mouse. Basic experiments revealed that scratching behavior at the site

of inflammation exacerbates contact dermatitis. In the future, if the mechanism of itching can be elucidated in detail and itching can be suppressed, dermatitis may be effectively suppressed.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflicts of interest.

Ethical Standards. All animal experiments were approved by the Animal Care Committee of the Hoshi University, Tokyo, Japan (permission code: 30-091).

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