HMGB1-mediated chromatin remodeling attenuates *Il24* gene expression for the protection from allergic contact dermatitis

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Dysregulation of inflammatory cytokines in keratinocytes promote the pathogenesis of the skin inflammation, such as allergic contact dermatitis (ACD). High-mobility group box 1 protein (HMGB1) has been implicated in the promotion of skin inflammation upon its extracellular release as a damage-associated molecular pattern molecule. However, whether and how HMGB1 in keratinocytes contributes to ACD and other skin disorders remain elusive. In this study, we generated conditional knockout mice in which the *Hmgb1* gene is specifically deleted in keratinocytes, and examined its role in ACD models. Interestingly, the mutant mice showed exacerbated skin inflammation, accompanied by increased ear thickening in 2,4-dinitrofluorobenezene-induced ACDs. The mRNA expression of interleukin-24 (IL-24), a cytokine known to critically contribute to ACD pathogenesis, was elevated in skin lesions of the mutant mice. As with constitutively expressed, IL-4–induced *Il24* mRNA, expression was also augmented in the *Hmgb1*-deficient keratinocytes, which would account for the exacerbation of ACD in the mutant mice. Mechanistically, we observed an increased binding of trimethyl histone H3 (lys4) (H3K4me3), a hallmark of transcriptionally active genes, to the promoter region of the *Il24* gene in the *hmgb1*-deficient cells. Thus, the nuclear HMGB1 is a critical "gate keeper" in that the dermal homeostasis is contingent to its function in chromatin remodeling. Our study revealed a facet of nuclear HMGB1, namely its antiinflammatory function in keratinocytes for the skin homeostasis.

HMGB1 | keratinocytes | IL-24 | dermatitis | H3K4me3

Allergic contact dermatitis (ACD) is a common inflammatory skin disease caused by irritants or low molecular weight allergens (haptens), such as chemicals and metal ions (1). These allergens penetrate the skin and trigger inflammatory immune responses (2). ACD reactions consist of two main phases: Sensitization and elicitation (3). Sensitization of allergens is caused by uptake of allergen by antigen-presenting cells, such as Langerhans cells and resident dendritic cells that subsequently migrate to the draining lymph nodes and present the contact allergens to naïve T cells (4). Contact allergen-specific T cells then proliferate and differentiate to effector T cells. The elicitation phase begins with repeated skin exposure with the same allergen, which brings about the recruitment of the effector T cells into the skin and the evocation of the reaction of ACD (4).

Although antigen-presenting cells that instruct T cell responses are fundamental for the pathogenesis of ACD, recent studies have demonstrated that keratinocytes are also critical for shaping the immune responses to contact allergens (5–7). Keratinocytes activated during sensitization phase induce proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and others, which trigger the activation of antigen-presenting cells and promotes T cell responses and expansion (2, 5, 8, 9). A critical contribution of keratinocyte-derived inflammatory mediators to the pathological immune responses has also been suggested in psoriasis, atopic dermatitis, and other skin disorders (10–12). Among many cytokines involved in ACD, the IL-10 family of cytokines has gained much attention, wherein IL-24 is one of the critical cytokines that exacerbate dermatitis (13, 14). In addition, damage-associated molecular pattern molecules released from inflamed or dying keratinocytes are implicated in the promotion of skin inflammation (15, 16).

High-mobility group box 1 (HMGB1) has two DNA-binding domains (A-box and B-box) and was originally described as a nuclear nonhistone chromosomal protein expressed constitutively

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Significance

High-mobility group box 1 protein (HMGB1) protein gained attention for its proinflammatory function in a number of pathological conditions, such as skin inflammation. In this study, a Cre/loxP-engineered mouse strain allowed for HMGB1 conditionally ablated in keratinocytes. When allergic contact dermatitis was induced in the HMGB1 mutant mice, skin inflammation was exacerbated in the mutant mice. Expression of interleukin-24 (IL-24), a proinflammatory cytokine associated to skin inflammatory disorders, was elevated in skin lesions of these mice. We also adduced evidence that that HMGB1 represses the binding of histone H3 (lys4) (H3K4me3) to the *Il24* gene promoter in keratinocytes. Therefore, our study revealed a facet of nuclear HMGB1 that represses the *Il24* gene for the suppression of allergic inflammatory skin disorders.

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in almost all cells, thus being classified as functioning to maintain nuclear homeostasis. HMGB1 is ubiquitously expressed, in contrast to its family members HMGB2, HMGB3, and HMGB4, which are restricted to particular tissues and organs (17–19). Much attention has also been focused on the extracellularly released HMGB1 as a mediator critical to inflammatory diseases, including skin inflammatory diseases (17, 18). According to the previous reports, HMGB1 release occurs during inflammation from damaged or dying cells, and extracellularly released HMGB1 transmits signals via innate immune receptors, such as Toll-like receptor 2 (TLR2), TLR4, and receptor for advanced glycosylation end products, which results in the amplification of inflammatory responses (17, 18). However, the relevance of these findings of extracellular HMGB1 remains untested in a broader immunological context.

Although recent studies have identified the correlation between elevated serum HMGB1 levels in patients with atopic dermatitis or psoriasis and the degree of disease severity (20, 21), contribution of HMGB1 in keratinocytes to inflammatory skin disorders has not been characterized well. In this study, we generated mice conditionally deficient in the *Hmgb1* gene in keratinocytes to investigate the in vivo role of HMGB1 in ACD and other skin inflammation. We show that, unlike the previous notion for the HMGB1's inflammatory role, HMGB1 functions in the nucleus of keratinocyte to suppress skin inflammation by a site-specific chromatin remodeling for the promoter region of *Il24* gene, the product of which critically contributes to ACD. We discuss our findings in terms of the versatile functions of HMGB1 in inflammatory diseases.

Results

Generation of Mice with Conditional Ablation of the *Hmgb1* Gene in Keratinocytes. To examine whether and how HMGB1 in keratinocytes contributes to skin inflammation, we first crossed *Hmgb1*-floxed mice (22) and transgenic mice carrying keratin 5 promoter-driven *Cre* gene (K5-Cre) (both in C57BL/6 background). Mice carrying *Hmgb1*-floxed and the *K5-Cre* genes (*K5-Cre*$^{+}$*Hmgb1*$^{f/f}$; K5-HMGB1 conditional knockout [cKO]) were born and grew without any abnormalities. HMGB1 deletion was confirmed in protein expression in epidermal keratinocytes obtained from K5-HMGB1 cKO mice (Fig. 1*A*) and immunohistochemistry in whole-ear skin (Fig. 1*B*). Although the K5 promoter is

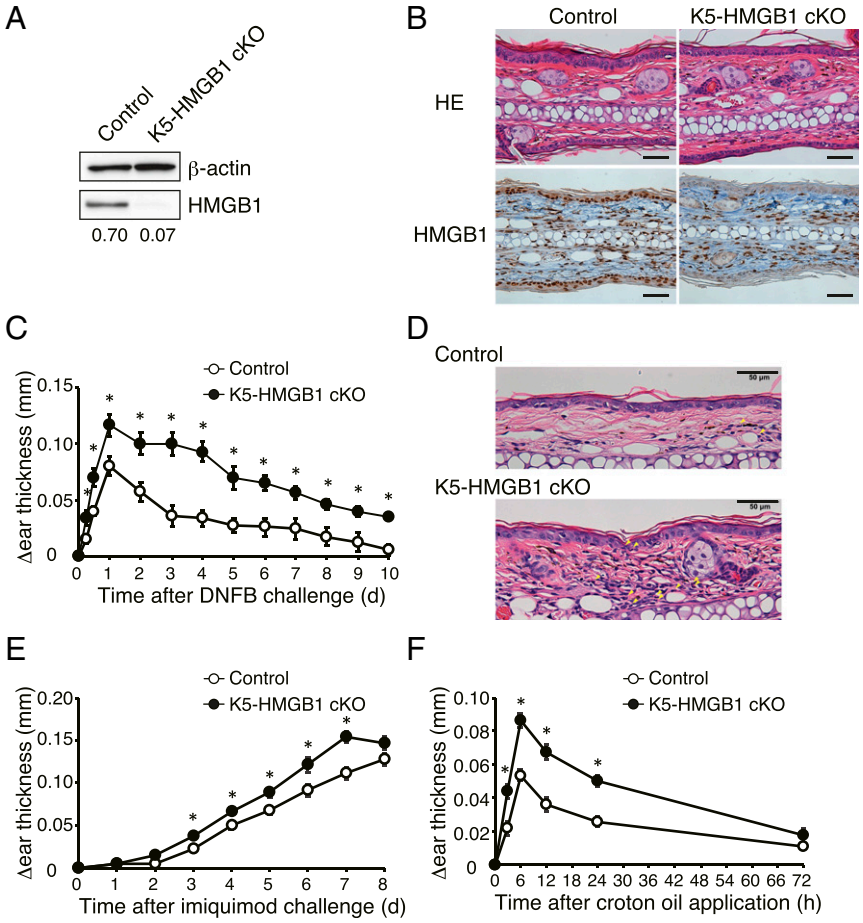

Fig. 1. Skin inflammation in mice selectively lacking HMGB1 in keratinocytes. (*A*) HMGB1 expression in epidermal keratinocytes. Keratinocytes prepared from epidermis of control (*Hmgb1*$^{f/f}$) or K5-HMGB1 cKO (*K5-Cre*$^{+}$*Hmgb1*$^{f/f}$) mice were subjected to immunoblot analysis. The relative band intensities of HMGB1 with respect to β-actin are shown. (*B*) Representative images of hematoxylin-eosin (HE) (*Upper*) and HMGB1 (*Lower*) staining of ear skin from control and K5-HMGB1 cKO mice. (Scale bars, 50 μm.) (*C*) Changes in ear thickness caused by repeated topical DNFB application. Control ($n = 8$) and K5-HMGB1 cKO ($n = 8$) mice were sensitized with 0.5% DNFB followed by challenge with 0.25% DNFB. The increase in ear thickness was measured after the DNFB challenge. (*D*) Histopathology of DNFB-elicited ear pinnae in control and K5-HMGB1 cKO mice. Ear samples were taken 24 h after elicitation and subjected to HE staining (original magnification, 200×). (Scale bars, 50 μm.) Representatives of five (control) and six (K5-HMGB1 cKO) mice in each condition. (*E*) Changes in ear thickness by imiquimod treatment. The increase in ear thickness in control ($n = 8$) and K5-HMGB1 cKO ($n = 10$) mice were measured after 5% Beselna cream treatment. (*F*) Changes in ear thickness by croton oil treatment. The increase in ear thickness in control ($n = 8$) and K5-HMGB1 cKO ($n = 12$) mice were measured after 2% croton oil treatment. Results shown are mean ± SEM, $^{*}P < 0.05$.

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also active in thymic epithelial cells (23), sizes and proportions of T cell population in the thymus and spleen from K5-HMGB1 cKO mice were unchanged (*SI Appendix*, Fig. S1*A*), indicating that the overall thymic T cell development occurs normally in the absence of HMGB1 in the epithelial cells.

Contribution of HMGB1 in Keratinocytes to ACD and Other Skin Inflammation. We next examined whether loss of HMGB1 in keratinocytes affects inflammation of ACD. We applied 2,4-dinitrofluorobenezene (DNFB), a low molecular weight hapten and inducer of ACD (24, 25) onto the ear of control (*Hmgb1*$^{f/f}$) and K5-HMGB1 cKO mice. Given the previous reports implicating the inflammatory role of HMGB1 (20, 21), we anticipated that HMGB1 deletion in keratinocytes would mitigate skin inflammation induced by DNFB treatment. However, we found that the ear swelling was significantly higher in K5-HMGB1 cKO mice compared to control mice (Fig. 1*C*). Histopathological analysis also showed more increased ear thickness and number of infiltrated lymphocytes in K5-HMGB1 cKO mice (Fig. 1*D*). These data suggest that HMGB1 in keratinocytes has a protective role in DNFB-induced ACD.

We further examined skin inflammation in K5-HMGB1 cKO mice using the oxazolone, a potent hapten. Ear thickness was measured before and after oxazolone treatment. Similar to the challenge with DNFB, the increase of ear thickness was significantly wider in K5-HMGB1 cKO mice compared to control mice (*SI Appendix*, Fig. S1*B*). A detailed time course of ear-swelling reaction by repetitive challenge of oxazolone was examined on days 0, 4, and 8. Both control and K5-HMGB1 cKO mice showed similar transition from delayed response on day 0 to immediate response on day 8, whereas the magnitudes of ear swelling reaction peak on days 0, 4, and 8 were significantly higher in K5-HMGB1 cKO mice than in control mice (*SI Appendix*, Fig. S1 *C*–*E*). Ear swelling induced by topical application of imiquimod, a TLR7 agonist, or croton oil was also assessed. Here again, the K5-HMGB1 cKO mice showed increased ear thickness compared to control mice (Fig. 1 *E* and *F*). These data are further indicative of HMGB1 suppressing the keratinocyte-mediated skin inflammation.

Characterization of the Inflammatory Skin Pathogenesis in K5-HMGB1 cKO Mice. We further characterized the skin inflammation of DNFB-induced ACD in the K5-HMGB1 cKO mice. First, we examined immune cells infiltrating into inflamed ear by flow cytometry analysis. After elicitation of ACD by DNFB, the population of CD11b$^+$Gr1$^+$ neutrophils, a major instigator of tissue inflammation, was significantly increased as compared to that of DNFB-untreated mice (Fig. 2*A*), an observation consistent with the increased ear thickness in K5-HMGB1 cKO mice (Fig. 1 *C* and *D*). Other immune cell populations, such as CD11b$^+$F4/80$^+$ macrophages, were not affected by *hmgb1* deletion (*SI Appendix*, Fig. S2*A*). Although it has been suggested that HMGB1 regulates autophagy activation during inflammation (26), no significant difference in the expression level of autophagy marker protein LC3 (isoforms I and II) was observed in the inflamed ear between control and K5-HMGB1 cKO mice (*SI Appendix*, Fig. S2*B*).

We also examined in which phase HMGB1 in keratinocytes is important for the suppression of ACD. To address this issue, we adoptively transferred lymphocytes from draining lymph nodes of DNFB-sensitized mice into recipient untreated mice (Fig. 2*B*), and then ear swelling was measured after DNFB treatment for elicitation. As shown in Fig. 2*B*, regardless of the type of donor mice, the increase of ear thickness was significantly higher when K5-HMGB1 cKO mice were used as recipient mice. There was no significant difference in ear thickness when lymphocytes from control or K5-HMGB1 cKO mice were transferred into control mice (Fig. 2*B*). Comparable ear swelling between control and K5-HMGB1 cKO mice were observed when these mice received DNFB to the ears without sensitization (*SI Appendix*, Fig. S2*C*). These data corroborate our findings in which HMGB1 in keratinocytes suppresses DNFB-induced inflammation during the elicitation phase.

Expression of Inflammation-Associated Cytokine Genes in the Absence of HMGB1. Next, we performed microarray analysis to identify genes whose expression was significantly affected by the lack of HMGB1 in keratinocytes. Perhaps expectedly from the above phenotypes, expression of several inflammation-related genes was found to be elevated in the whole ear of K5-HMGB1 cKO mice compared to that of control mice (*SI Appendix*, Fig. S2*D*). qRT-PCR analysis also verified significantly increased expression of mRNAs for IL-1β, IL-6, IL-24 (also known as FISP; IL-4–induced secreted protein), and others (Fig. 2*C*). Elevated expression of mRNA for CXCL1, a potent neutrophil chemoattractant, was also found (Fig. 2*C*), which is consistent with the higher infiltration of neutrophils in the ear of K5-HMGB1 cKO mice (Fig. 2*A*).

We further examined inflammatory cytokine gene expression in imiquimod-induced psoriasis and a croton oil-induced irritant contact dermatitis models (*SI Appendix*, Fig. S2 *E* and *F*), which showed increased inflammation in K5-HMGB1 cKO mice (Fig. 1 *E* and *F*). Notably, we found that *Il24* mRNA expression was increased in the ear of K5-HMGB1 cKO mice in both models, while the dependence of HMGB1 on the expression of other mRNAs described above varied among the disease models (*SI Appendix*, Fig. S2 *E* and *F*). These results therefore suggest that HMGB1 selectively regulate the *Il24* gene.

Considering that high expression of *Il24* mRNA in K5-HMGB1 cKO mice in the ACD model and numerous reports showing the pathogenic role of IL-24 in ACD and other skin inflammation (14, 27, 28), it is likely that the elevated expression of *Il24* mRNA in keratinocytes of the ear accounts, at least in part, for the exacerbated skin inflammation in K5-HMGB1 cKO mice. In this context, it has been known that IL-24 induces acanthosis and production of chemokines, thereby triggering a neutrophil influx in ACD (13, 14).

Increased *Il24* mRNA Expression in Keratinocytes with HMGB1 Depletion. The above findings suggested HMGB1 regulates *Il24* mRNA expression in keratinocytes. To examine this point further, we isolated the epidermis, in which keratinocytes are predominant, from the ear of control or K5-HMGB1 cKO mice after development of DNFB-induced ACD and examined mRNA expression for inflammatory cytokines by qRT-PCR analysis. Notably, *Il24* mRNA levels were significantly higher in the epidermis from K5-HMGB1 cKO mice as compared with the control mice (Fig. 3*A*), supporting the notion that HMGB1 negatively regulates its gene expression in keratinocytes.

The above observations prompted us to closely examine keratinocytes in the context of HMGB1-dependent regulation of *Il24* mRNA expression. We then cultured primary keratinocytes from control and K5-HMGB1 cKO mice. Since it has been reported that IL-4, IL-17A, or IL-1β stimulation induces *Il24* mRNA in various types of cells (29, 30), their stimulatory effects on *Il24* mRNA expression in stimulated primary keratinocytes was assessed. Among these cytokines, IL-4, the cytokine associated with Th2-type T cell response, most markedly induced *Il24* mRNA in primary keratinocytes, and that the induction was further enhanced in the cells from K5-HMGB1 cKO mice (Fig. 3*B*). Interestingly, constitutive expression of *Il24* mRNA was also elevated in *Hmgb1*-deficient primary keratinocytes (Fig. 3*B*), suggesting a mechanism by which HMGB1 regulates the *Il24* gene expression independent of cytokine signals.

We further examined IL-4–induced gene-expression profiles in *Hmgb1*-deficient primary keratinocytes by microarray and

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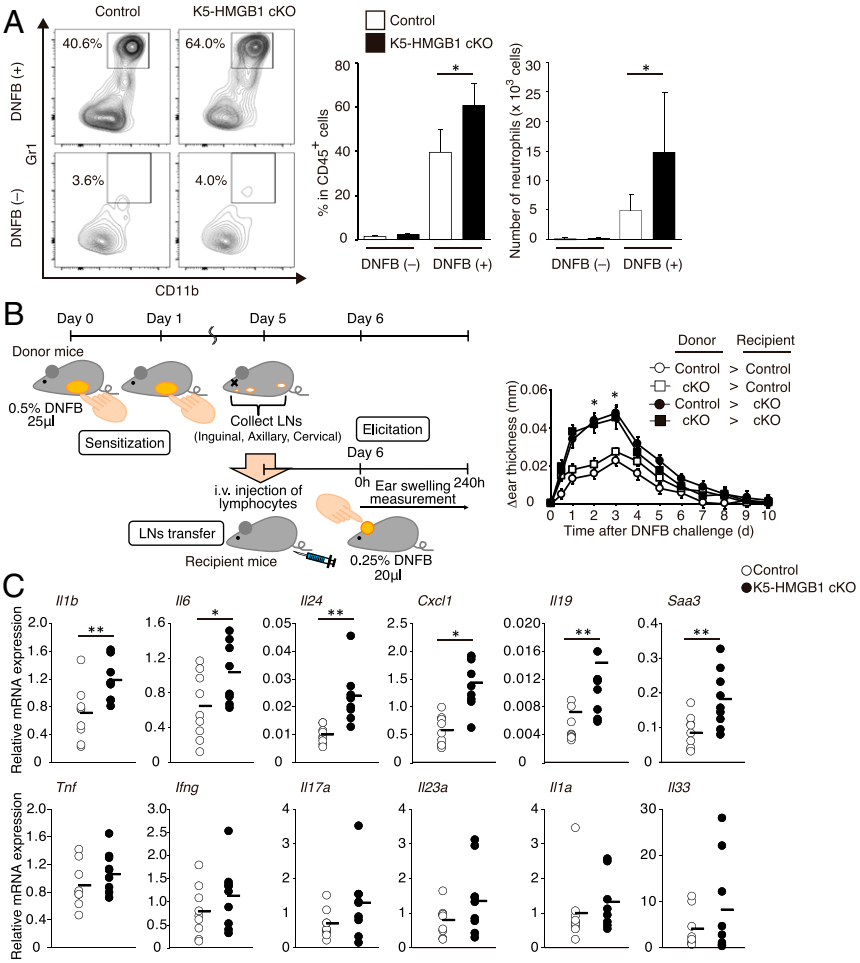

Fig. 2. Characterization of increased skin inflammation of DNFB-induced ACD in K5-HMGB1 cKO mice. (*A*) Infiltration of neutrophils in DNFB-treated ear of control and K5-HMGB1 cKO mice. Control and K5-HMGB1 cKO mice were sensitized with 0.5% DNFB followed by challenge with 0.25% DNFB. Ear samples were taken 24 h after elicitation and subjected to flow cytometry analysis. $CD11b^{+}Gr1^{+}$ neutrophils in $CD45^{+}$ cells were gated and the percentages of neutrophils were analyzed (*Left* and *Center*). The number of infiltrated neutrophils are also shown (*Right*). (*B*) The DNFB-induced ACD model using chimeric mice generated by transplantation of lymphocytes from draining lymph nodes (LNs) of DNFB-sensitized mice (*Left*). Lymphocytes from DNFB-sensitized control or K5-HMGB1 cKO mice were used as donor cells. Control and K5-HMGB1 cKO mice were sensitized with 0.5% DNFB at first 2 d. Five days later, draining lymph nodes were collected to prepare lymphocytes suspension; 5×10^6 lymphocytes were intravenously transplanted into control or K5-HMGB1 cKO recipient mice. One day after transfer, the ears of chimeric mice were treated with 0.25% DNFB for elicitation. The increase in ear thickness was measured after elicitation (*Right*). (*C*) qRT-PCR analysis for mRNA expression in the ear. Control ($n = 9$) and K5-HMGB1 cKO ($n = 8$) mice were sensitized with 0.5% DNFB followed by challenge with 0.25% DNFB. Total RNA of the ear samples was extracted 24 h after elicitation and subjected to qRT-PCR analysis. Results shown are mean ± SEM, $*P < 0.05$; $**P < 0.01$.

qRT-PCR analysis. Remarkably, *Il24* mRNA was the most up-regulated by IL-4 stimulation in the control cells, which was further enhanced in *hmgb1*-deficient cells (*SI Appendix*, Fig. S3). Of note, some IL-4–induced genes, including *Atp1b1* and *Dusp4*, were not enhanced in *Hmgb1*-deficient cells (*SI Appendix*, Fig. S3). These results further support the notion that HMGB1 selectively exerts it suppressive functions on *Il24* gene.

Altered Chromatin Modification in *Hmgb1*-Deficient Keratinocytes. To gain further insights into the molecular mechanism underlying the enhancement of *Il24* mRNA expression by the HMGB1 deficiency, we newly generated a mouse keratinocyte cell line PAM212 with the *Hmgb1* gene ablated using CRISPR/Cas9 (*SI Appendix*, Fig. S4*A*). Similar to the results in *Hmgb1*-deficient primary keratinocytes (Fig. 3*B* and *SI Appendix*, Fig. S3), *Il24* mRNA expression was higher in the *Hmgb1*-deficient PAM212 cells as compared to control cells upon IL-4 stimulation (Fig. 4*A*). Expectedly, reexpression of HMGB1 in *Hmgb1*-deficient cells resulted in attenuation of *Il24* promoter activation by IL-4 (*SI Appendix*, Fig. S4*B*).

Next, we assessed IL-4–induced tyrosine phosphorylation of STAT6 (Tyr641), a hallmark of its activation (31). As shown in Fig. 4*B*, activation of STAT6 was normal in *Hmgb1*-deficient cells. Consistent with this result, we found no overt abnormalities in mRNA expression levels of known negative regulators for STAT6 activation (*SI Appendix*, Fig. S4*C*). These results indicated that HMGB1 does not affect IL-4 signaling. We further examined the intracellular localization of HMGB1 and found that it remains in the nucleus even after IL-4 stimulation (*SI Appendix*, Fig. S4*D*). Thus, nuclear HMGB1 is likely responsible for the suppression of *Il24* mRNA expression.

The above observations prompted us to perform chromatin immunoprecipitation sequencing (ChIP-seq) analysis to ask the nuclear role of HMGB1 in *Il24* mRNA expression. Since HMGB1 binds to the minor groove of DNA (32, 33), we first searched HMGB1-binding sites on the *Il24* gene (*SI Appendix*, Fig. S5*A*). However, we could not find any of them on the *Il24*

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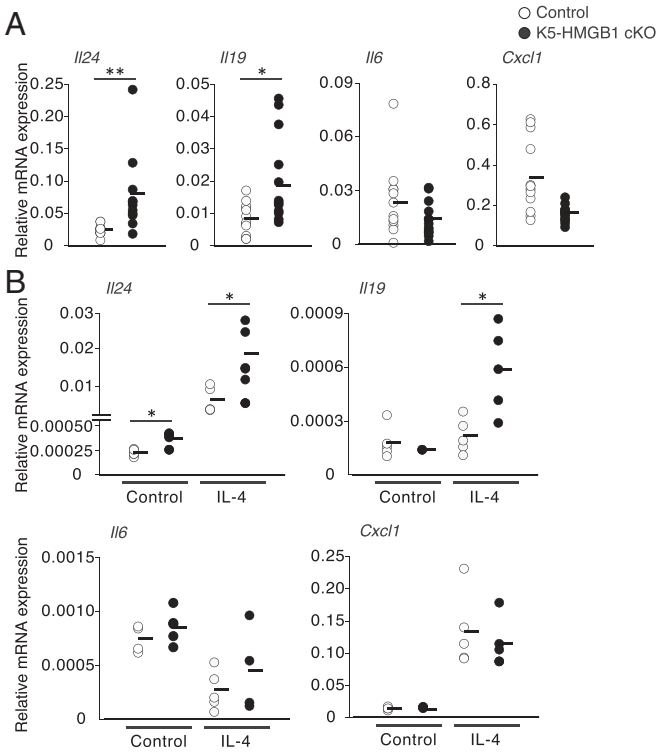

Fig. 3. Expression of *Il24* mRNA in *Hmgb1*-deficient primary keratinocytes. (*A*) Cytokine mRNA expression in epidermis by DNFB challenge. Control (n = 12) and K5-HMGB1 cKO (n = 12) mice were sensitized with 0.5% DNFB followed by challenge with 0.25% DNFB. Total RNA of epidermis was extracted 24 h after elicitation and subjected to qRT-PCR analysis. (*B*) Cytokine mRNA expression in primary keratinocytes by IL-4 stimulation. Control (n = 5) and *Hmgb1*-deficient (n = 5) primary keratinocytes were stimulated IL-4 (100 ng/mL) for 12 h. Total RNA was prepared and subjected to qRT-PCR analysis. Results shown are mean ± SEM, $^*P < 0.05$.

gene, while enriched ChIP DNA fragments were detected on other genes, such as the *Sntb2* gene (*SI Appendix*, Fig. S5*B*). We further examined histone markers, such as trimethylation of histone H3 lysine 4 (H3K4me3), which is associated with open chromatin state and active transcription (34, 35). As shown in Fig. 4*C*, the histone marker was found in two regions, one near the transcription start site of the *Il24* gene (R1) and the other at the distal site (R2). ChIP-qPCR analysis revealed increased H3K4me3 marks in the R1 region in *Hmgb1*-deficient cells, both with and without IL-4 stimulation (Fig. 4*C*). This may account for the reason why expression of *Il24* mRNA is high in *Hmgb1*-deficient keratinocytes. Perhaps expectedly, the ChIP-seq analysis also revealed significant changes in H3K4me3 marks (3,646 peaks) over the whole genome in the cells (*SI Appendix*, Fig. S5*C*). Collectively, these data are consistent with previous reports that indicate HMGB1 is a critical regulator of epigenetic complex that regulates proinflammatory and related pathogenic gene expression (19, 36, 37).

Discussion

Although the proinflammatory role of extracellular HMGB1 in skin inflammation has been suggested (20, 21), the in vivo involvement of HMGB1 in keratinocytes remained obscure heretofore. In this study, we examined the HMGB1's role in keratinocyte by generating cKO mice in which *Hmgb1* is selectively ablated in keratinocytes in the context of ACD. We found that, in all dermatitis models we tested, K5-HMGB1 cKO mice showed enhanced ear swelling (Fig. 1 *C* and *D* and *SI Appendix*, Fig. S1 *B–E*) accompanied by elevated *Il24* mRNA expression (Fig. 2*C* and *SI Appendix*, Fig. S2 *E* and *F*). We have also seen high *Il24* mRNA expression in keratinocyte-rich epidermis of K5-HMGB1 cKO mice (Fig. 3*A*). Therefore, the elevated expression of *Il24*

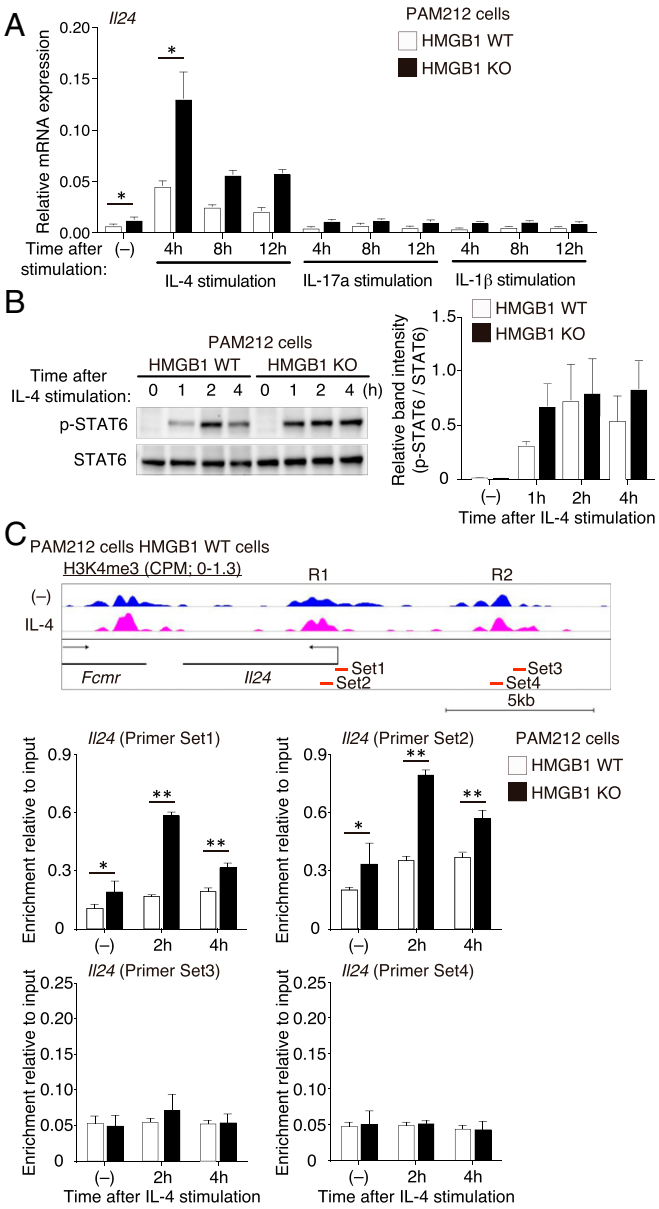

Fig. 4. Histone modification H3K4me3 in *Hmgb1*-deficient PAM212 keratinocyte cell line. (*A*) Expression of *Il24* mRNA expression in *Hmgb1*-deficient PAM212 cells. Control and *Hmgb1*-deficient cells were left untreated or stimulated by IL-4 (100 ng/mL), IL-17A (100 ng/mL), or IL-1β (100 ng/mL) for the indicated time periods. Total RNA was prepared and subjected to qRT-PCR analysis. (*B*) STAT6 phosphorylation by IL-4 stimulation in *Hmgb1*-deficient PAM212 cells. Control and *Hmgb1*-deficient cells were left untreated or stimulated by IL-4 (100 ng/mL) for the indicated time periods. Cell lysates were prepared and subjected to immunoblot analysis using anti-phospho STAT6 (Tyr641) or STAT6 antibodies. (*C*) Increased H3K4me3 mark on the *Il24* gene locus in *Hmgb1*-deficient cells. Control and *Hmgb1*-deficient cells were left untreated or stimulated by IL-4 (100 ng/mL) for the indicated time periods, followed by ChIP-seq analysis using anti-H3K4me3 antibody. Two H3K4me3 peak regions (R1 and R2) on the *Il24* gene locus are shown (*Upper*). ChIP-qPCR analysis was performed using specific primer sets (*Upper*) for R1 and R2 regions (*Lower*). Data are shown as means ± SEM, $^*P < 0.05$; $^{**}P < 0.01$.

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mRNA in keratinocytes of the mutant mice accounts, at least in part, for the exacerbation of ACD. On the other hand, our study does not rule out the possibility that other proinflammatory cytokines, including IL-1β and IL-6, elevated in DNFB-treated mutant mice (Fig. 2*C*), also contribute to the pathogenesis, although their expression varies between ACD models (*SI Appendix*, Fig. S2 *E* and *F*). Generation of mice with keratinocyte-specific ablation of *Il24* and *Hmgb1* genes is an interesting future issue.

IL-24 is a cytokine of IL-10 superfamily and has gained much attention as a key cytokine for ACD and other inflammatory diseases (13, 14, 38, 39). Unlike IL-10, however, IL-24 is inflammatory and induces neutrophil recruitment and acanthosis of the skin in ACD (13, 14). Indeed, it has been reported that *Il24*-transgenic mice show ACD phenotype, whereas *Il24*-deficient mice are protected from ACD (14, 40). It is induced by stimulation of cytokines, such as IL-4 in natural killer cells, macrophages, and keratinocytes (29, 30). Therefore, increased expression of *Il24* mRNA, observed here will likely account for the exacerbation of ACD in K5-HMGB1 cKO mice. Although IL-24 is known to be produced by other cell types, such as monocytes and T and B cells, our results indicate the critical role of keratinocyte production of IL-24 in ACD. Whether HMGB1 regulates *Il24* gene in the above-mentioned cell types, showing a phenotype similar to what we observed here in the context of ACD, remains to be clarified.

Adoptive transfer experiment also showed loss of HMGB1 in keratinocytes worsened inflammation of ACD during the elicitation phase in which keratinocytes are activated by inflammatory mediators released by surrounding cells. Although further work is needed to clarify the precise induction mechanism of the *Il24* gene regulation during ACD, we surmise that *Il24* mRNA is induced in the activated keratinocytes occurs during the elicitation phase. It is likely that IL-4 signaling, which induces the *Il24* gene (Fig. 3*B*) in keratinocytes (and perhaps other cells), is involved in the activation of keratinocytes. In fact, reduced skin inflammation in DNFB-induced ACD has been reported in *Il4*-, *Il4ra*-, and *Stat6*-deficient mice (41–43). Nonetheless, the issue of whether HMGB1–IL-24 pathway operates via IL-4 signaling in our ACD models needs further investigation.

In further exploring the mechanism of HMGB1-mediated *Il24* gene suppression in keratinocytes, large-scale changes in histone modification such as H3K4me3 were observed in *Hmgb1*-deficient mouse keratinocytes (*SI Appendix*, Fig. S5*C*). The H3K4me3 marker was increased in the promoter region containing a transcription start site of the *Il24* gene in the cells (Fig. 4*C*), which we surmise might lead to a more potentiated transcription of *Il24* mRNA. Consistent with this, even in the absence of IL-4 stimulation, we observed a notable and constitutive *Il24* mRNA expression in both *Hmgb1*-deficient primary keratinocytes and keratinocyte cell line (Figs. 3*B* and 4*A*). These data further underscore the notion that HMGB1 functions to attenuate *Il24* gene expression by chromatin remodeling (i.e., by the suppression of H3K4me3 binding to the *Il24* promoter region). From these data, we infer that HMGB1 consequently interferes with the biding of transcription factors, such as c-Jun, C/EBP-β, and others that are involved in the *Il24* gene transcription (39, 44).

How HMGB1 regulates histone modification in particular chromatin regions remains to be clarified. Given that HMGB1 binds to genomic DNA (32, 33), we envisaged that HMGB1 serves as a scaffold for the binding of histone-modifying enzymes, which may lead to the maintenance of appropriate histone modifications. Our ChIP-seq analysis using anti-HMGB1 revealed faint ChIP signals on some genes, such as the *Sntb2* gene (*SI Appendix*, Fig. S5*B*). However, we could not detect apparent HMGB1 signal in the promoter region of the *Il24* gene (*SI Appendix*, Fig. S5*A*). Nevertheless, we cannot exclude the possibility of HMGB1 binding to the promoter since recent reports showed that HMG-box DNA-binding domains are highly sensitive to formaldehyde fixation (45, 46), which is commonly used in the ChIP experiment. This makes it difficult to release immunoprecipitated DNA and leads to weak ChIP signals. Therefore, new technologies or more efficient tools, such as high-affinity HMGB1 antibodies, might be needed to clarify further the transcriptional interaction between HMGB1 and the *Il24* gene.

In conclusion, our study revealed an unprecedented role of HMGB1 in the maintenance of histone modifications for the *Il24* gene transcription, thereby playing a protective role for ACD. Loss of HMGB1 in keratinocyte broadly changes transcriptional activity and exaggerated skin inflammation in mice. Studies of HMGB1 cKO mice in which the gene is selectively deleted in other cell types, such as immune cells, may reveal new aspects of how HMGB1 contributes to other inflammatory and other types of diseases.

Materials and Methods

Mice. *Hmgb1*$^{flox/flox}$ mice were generated previously (22). *Keratin5* (K5)-Cre transgenic mice were kindly provided by Junji Takeda, Osaka University, Osaka, Japan (47). C57BL/6 mice were purchased from CLEA Japan Inc. All animal experiments were done in accordance with guidelines of the University of Tokyo.

Reagents and Cells. Recombinant mouse IL-4, IL-17, and IL-1β were purchased from Peprotech. Cells from the thymus, spleen, and lymph nodes were prepared and analyzed as previously described (48). Mouse keratinocyte cell line PAM212 cells were kindly provided by Kiyoshi Nishioka, Tokyo Medical and Dental University, Tokyo, Japan, and maintained in Eagle's minimum essential medium (Thermo Fisher Scientific) supplemented with 10% FCS (Sigma).

Additional information can be found in *SI Appendix*, *SI Materials and Methods*.

Data Availability. Microarray analysis and ChIP-seq analysis data have been deposited in the Gene Expression Omnibus (GEO) database (accession no. GSE159695) and Sequence Read Archive (accession no. DRA010946), respectively. All raw data are available from the corresponding authors upon reasonable request.

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